THE INTESTINAL ABSORPTION OF MACROMOLECULES IN ADULT MICE

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ABSTRACT

The intestinal absorption of 2 bacterial antigens was studied in adult mice. These were flagellin (FLA), purified from <u>Salmonella adelaide,</u> and Boivin Antigen (BA), extracted from <u>Vibrio cholerae.</u> With the latter, 4 techniques were used, which demonstrated that small amounts of macromolecular BA were absorbed. These include the use of (1) I^{125}-labelled antigen <u>in vivo;</u> (2) H^3DNP-labelled antigen <u>in vivo;</u> (3) I^{125} specific antibody injected i.v. to detect cold, absorbed antigen <u>in vivo;</u> and (4) everted gut sacs. The use of I^{125} antigens in the intact mouse and rat (but not in everted sacs) was discredited by our studies. On the other hand, H^3 DNP was a stable and suitable tag. Using this system, we observed a marked difference between the absorption of BA and FLA, which was consistent with the observed fate of these antigens following their i.v. administration, and their relative degradabilities in intestinal juice.

Oral immunization inhibited the intestinal absorption of the specific antigen in the above models. The efficiency of this was generally low, but significant. This function may be a prerogative of IgA antibodies, since serum antibodies were found to induce intestinal anaphylaxis, as manifested by death or increased gut permeability.

The absorption of immunogenic BA was also demonstrated in mice. This was shown indirectly by the ability of oral doses of the antigen to prime an animal for an anamnestic, systemic response (on i.v. boosting), and directly by the ability of absorbed material recovered from the plasma <u>in vivo</u> or everted gut sacs to

prime normal mice when injected i.v.. In all cases, the resultant, humoral response in the recipient animals was qualitatively different (presumably due to IgA production) from that obtained in mice primed i.v. with the native antigen, and the significance of this is discussed.

STATEMENT

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

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"I am convinced digestion is the great secret of life; and that character, talents, virtues and qualities are powerfully affected by beef, mutton, pie-crust and rich soups."

SYDNEY SMITH (1771 - 1845)

letter to Arthur Kinglake
Sept. 30, 1837.

CONTENTS

1. CHAPTER 1. INTRODUCTION

Following the discovery of IgA by Heremans and his colleagues in 1959 and the subsequent demonstration by Tomasi et al (1965) that this immunoglobulin predominated in body secretions, the biological importance of IgA in immunological reactions occurring in the gastrointestinal tract became appreciated.

In consequence, the gut is now thought to possess a relatively discrete, immunological role in addition to those of digestion and absorption, and that of a physical barrier between the body tissues and the outside world. However, since there is increasing evidence that intact macromolecules are absorbed through the gut, the efficiency of the barrier function has been recently questioned. In this regard, the following quotation from Verzar and McDougall (1936) appears to be relevant: "The quantities of undigested proteins which are absorbed must generally be extremely small, and the absorption of natural, unchanged proteins therefore cannot be looked on as the physiological way of absorption, but rather as a failure (which occurs almost constantly) of the normal conditions of intestinal function".

1.1 Historical Perspectives on Protein Absorption

When considering the absorption of antigenic (macromolecular) material from the gut, it is pertinent to examine the development of ideas related to the intestinal absorption of proteins (which has been intensively studied for a long time), since these are facets of the same phenomenon.

The early popular notions regarding the form in which protein was absorbed, as listed by Van Slyke and Meyer (1913) were:-

1. The ingested proteins are absorbed and incorporated into the body without undergoing any marked chemical change.

2. The food proteins are first hydrolysed in the alimentary tract; the products of digestive hydrolysis are then absorbed into the blood and carried to the tissues.

3. The products are deaminized in the wall of the intestine before entering the circulation.

4. The products are synthesized into serum protein before entering the circulation. The serum proteins thus formed serve as nourishment for the tissues in general.

The idea that proteins were absorbed intact was probably the earliest (formulated in the early 19th Century), before proteolytic enzymes were known. Even when "peptone" was later known to be a product of protein digestion by gastric juice (Prout 1824), pepsin (Schwann 1836) and trypsin (Kuhne 1867), the failure to find this product in the circulation was taken to support the concept of whole protein uptake. This idea was further encouraged by finding protein in the urine when egg albumin was eaten (see Van Slyke and Meyer 1913). However, with the discovery of "erepsin" by Cohnheim (1901) - which could further digest peptones to amino-acids - the idea of intact protein absorption became less favoured, particularly when Abderhalden (1912) showed that amino-acids could maintain the nitrogenous equilibrium and growth of dogs just as well as intact proteins.

After proteins, the absorbed materials were presumed to be

peptones, proteoses or polypeptides, as these were more diffusible and disappeared more rapidly from the intestine than whole protein. However, for several decades, there was no positive proof of absorbed peptone (see Verzar & McDougall 1936; Matthews 1977).

The important discovery of "erepsin" implied that amino-acids were the "currency" of absorbed proteins. Though Abderhalden (1912) found all the known amino-acids in the mammalian intestine during the absorption of proteins, and together with London (1910) demonstrated that these amino-acids rapidly disappeared from the intestine (80% in 3 hours), there was great difficulty in demonstrating absorbed amino-acids in the circulation. Thus, as late as 1905, Kutscher and Seemann failed to detect amino-acids in the blood of dogs fed with protein, despite excluding the liver and kidneys from the circulation. The failure to detect absorbed amino-acids in the circulation on the one hand, prompted the idea that these amino-acids were deaminated during transport through the wall, and on the other, supported the long-standing theory (since 1870) that the proteolytic products of protein digestion were re-synthesized into protein in the intestinal wall. The former theory was refuted by Folin and Denis (1912) who, using their new sensitive technique for determining ammonia and urea, found no increase in these compounds during the absorption of alanine. The latter theory was disproved by the positive findings of increased non-protein nitrogen in the circulation during absorption (Cathcart and Leathes 1905; Folin and Denis 1912). Furthermore, Van Slyke and Meyer (1913), using their new, sensitive nitrous acid method for detecting amino-acids, not only observed a 2-fold increase in the amino-nitrogen in the blood of

dogs following the ingestion of alanine or whole protein, but also showed that intravenously injected amino-acids rapidly disappeared from the circulation into all tissues. The liver in particular, could accumulate 150mg % of the amino-acids, and then rapidly catabolise these. Thus, the rapid clearance of amino-acids from the circulation could account for previous failures in detecting absorbed amino-acids using less sensitive techniques. The important conclusion thus reached by Van Slyke and Meyer in 1913 was: "Ingested proteins are hydrolysed in the digestive tract setting free most, if not all, of their amino-acids. These are absorbed into the blood stream, from which they rapidly disappear as the blood circulates through the tissues." This concept was essentially maintained in the "classical hypothesis" of protein absorption, which prevailed for the next fifty years, i.e. that protein was completely hydrolysed to amino-acids in the gut lumen (by pepsin, the pancreatic proteolytic enzymes and erepsin (enzymes) of the "succus entericus"), which were then absorbed into portal blood (Matthews 1977). In support of this, Christensen et al (1947) studying the absorption of gelatin in the peripheral plasma of man, and Dent and Schilling (1949), using their new 2-dimensional paper chromatography on the portal and jugular blood of dogs fed a protein meal, detected increases in amino-acid concentration but little or no increase in peptide concentration. Wiseman's finding (1951) that amino-acids were actively transported also lent support to the hypothesis.

A change in our understanding of protein absorption came with the findings of Newey and Smyth (1960), Matthews et al (1968), and others (summarised by Ugolev et al 1977) that intraluminal hydrolysis of proteins was incomplete, and that small peptides

could also be actively taken up by the mucosal cells before
further hydrolysis (membrane or intracellular) to amino-acids and
entry into portal blood. Thus Craft et al (1968) showed that in
man, glycine was absorbed more rapidly from its di- and tripeptide
than from an equivalent, equimolar quantity of free glycine.
These studies together with the observations that patients with
inherited defects in amino-acid absorption (Hartnup disease and
cystinuria) suggested separate transport processes for free and
peptide-bound amino-acids. It was later found that some peptides
were not totally hydrolysed during transport. Thus, Prockop et al
(1962) found that about 10% of proline-hydroxyproline was excreted
in the urine of man after a gelatin meal, and Peters et al (1970)
found 4-8% glycyl-glycine in the portal blood of rats fed with
glycine peptides. Our current understanding of protein absorption
is adequately summarised by Gray and Cooper (1971): "The initial
stage of digestion of dietary protein is accomplished by the
sequential action of activated pancreatic proteases within the
intestinal lumen, yielding about 30% neutral and basic amino-acids
and 70% small peptides. The amino-acids are transported by their
specific mechanisms. The oligopeptides are probably hydrolysed by
brush border enzymes on the intestinal surface (particularly those
peptides containing mainly neutral amino-acids) and the released
amino-acids transported, or they enter the intestinal cell intact
(glycine, proline-hydroxyproline, dicarboxylic type peptides) to
be hydrolysed by soluble intracellular enzymes. Approximately 10%
of peptides containing predominantly glycine or proline -
hydroxyproline enter and exit from the intestinal cell
unhydrolysed".

The finding that some dipeptides were absorbed intact has

prompted much investigation on their quantitation, not only in mammals, but also in microorganisms (Payne 1977) and plants (Enori and Mikola 1977). From a nutritional point of view, dipeptide absorption may be important (and certainly economical), particularly with the recent findings of Adibi et al (1977) that the dipeptides, glycyl-leucine and glycyl-glycine, when intravenously given to rats were not excreted but were rather, rapidly metabolised to their constituent amino-acids by the body tissues, particularly the kidneys and the intestine. The rapid disappearance of these dipeptides from circulation obviously poses problems for their detection in the circulation after oral absorption.

The question of whether the larger oligopeptides were absorbed has also been examined. Burston et al (1977) found that the tri-peptide (gly-sar-sar) was less readily taken up in their everted hamster ring model than the di-peptide (glycylsarcosine) and the tetra-peptide (gly-sar-sar-sar) was not taken up at all. Similar findings were reported by Adibi and Morse (1976) with tetraglycine. Although there is a paucity of data in this area using direct biochemical approaches,the more sensitive immunological methods are providing evidence, that macromolecules, even as large as bovine serum albumin, are absorbed intact. Though the amount is generally small and nutritionally unimportant, it is nevertheless immunologically significant. (This aspect is further discussed in 1.3.)

It has however been known for a long time that the newborn of many mammals can absorb large quantities of intact proteins up to a certain age and this is discussed in the next section, 1.2.

1.2   Macromolecular Absorption in the Neonate

Up to a certain age, absorption of macromolecules from the gut has been demonstrated in many mammalian neonates. These include man (Rothberg 1969), monkey (Lev and Orlic 1973), calf (Howe 1921; Smith and Little 1924), pig (McLance and Widdowson 1957), goat (Comline 1951), lamb (Charlwood and Thomson 1948; McCarthy and McDougall 1953), dog (McLance et al 1949), chicken (Porter et al 1977), rat (Halliday 1954) and mouse (Clark 1959). This topic has been well reviewed by Walker and Isselbacher (1974).

In the ruminants, which receive all their maternal immunoglobulins orally in the postpartum period, the period of increased permeability after birth is short, being 24-72 hours in the calf (Smith and Little 1924; Deutsch and Smith 1957) and 24-29 hours in Lamb (Charlwood and Thomson 1948; McCarthy and McDougall 1953). Following the ingestion of colostrum by the newborn calf, globulin was detected in both blood (Howie 1921) and urine (Smith and Little 1924), and proteinuria was evident during the first 3 days (Smith and Little 1924). In the pig, the serum protein rose from 2.3gm % at birth (and at 40 hours when fed water only) to 5.3gm % when fed with sow's milk plus colostrum (McLance and Widdowson 1959). In the sera of the neonatal pig, calf and chicken, the predominant immunoglobulin class absorbed is IgG (Porter et al 1977). Whereas in the neonatal pig, maternal secretory IgA is left to function in the intestinal lumen and very little is absorbed into circulation (Porter et al 1970), in the calf, high levels of it may be absorbed, which is however, lost very rapidly to the tissues (Porter 1972).

While globulin appeared to be absorbed entirely into the lymph of the newborn calf and goat (Comline et al 1951), appreciable amounts of intact albumin were detected in portal blood (Balfour and Comline 1959).

The factors which bring this brief period of macromolecular absorption to an end in ruminants are unknown. Whilst Lecce et al (1964) have suggested a controlling effect from colostrum factors, Deutsch and Smith (1957) were unable to prolong this period by feeding a milk-free diet.

In the rodents, which derive passive immunity partly from the intrauterine transport of maternal immunoglobulins, and partly from postpartum absorption, the period of increased permeability is prolonged (this is 21 days in the rat; Halliday 1954) and the uptake is more selective than with the ruminants. Thus, not only is globulin absorbed more than albumin, but the homologous rat globulin is taken up better than other heterologous globulins. Halliday (1954-1955) detected rat and mouse globulin in the circulation of suckling rats fed these proteins within 30 minutes, with a maximum absorption at 3 hours; however, rabbit globulin was absorbed more slowly, and that from the cow and fowl probably not at all. This specificity appeared to reside in the Fc portion of IgG (see Waldman and Jones 1976) which supported Brambell's theory (1966) that globulins were protected from degradation during transport across yolk sac and intestinal epithelium, by their attachment to specific receptors. Rodewald (1970) and Jones and Waldman (1972) further showed that this specialized transport was localized in the jejunum. On the other hand, with other

proteins, the transport is apparently non-selective; while Garney (1968) showed greater uptake of ferritin in the ileum of suckling rats, Walker et al (1972b) demonstrated similar absorption rates of horseradish peroxidase in both the jejunum and ileum. The termination of this absorptive period is associated with the maturation of the epithelial cells (Rodewald 1973), which can be mimicked by corticosteroids (Halliday 1959).

The literature on neonatal absorption in man is scarce. There appears to be little absorption of globulins (Iyengar and Selveraj 1972). This is not surprising in view of the fact that intrauterine transport of maternal antibodies (see Balfour and Jones 1976) is an important source of passive immunity, and furthermore, human colostrum contains predominantly secretory IgA (Chodirker and Tomasi I963) (unlike those of rodents and ruminants, where IgG predominates) which is less readily transported across the epithelium (Porter 1969). The evidence for the increased absorption of other proteins in the infant is indirect; Rothberg and Farr (1965) found higher anti-dietary antibodies in children than in adults. However, the decreased levels in adults may also be due to orally induced tolerance (Korenblat et al 1968).

The mechanism of macromolecular absorption in the neonate is thought to be an active process (Lecce 1966). The macromolecules are endocytosed, transported in membrane bound vesicles (phagosomes) which later fuses with lysosomes, whence intracellular digestion begins, and those molecules that escape breakdown are then extruded from the other side of the columnar cell (Walker and Isselbacher 1974; Allison and Davies 1974).

Thus, intracellular digestion is an important factor in determining the specificity of the transport process and the rates at which different proteins are absorbed. In fact, an increased lysosomal activity during maturation may account for the decreased uptake of macromolecules in the adult.

In short, a brief period of increased macromolecular uptake from the neonatal gut in mammals appears universal. This absorption is specific with regards to immunoglobulins in some species, but is generally non-specific with other macromolecules.

1.3   Evidence of Macromolecular Absorption from the Adult Gut

Since 1900, observations have been made on the absorption of macromolecules from the intestine of adult animals. For instance, Hirsch (1906) found whole starch grains in the blood and urine of an animal fed raw starch, and Fischer (1931) found yeast cells in the portal blood and tissues (viz. the liver) of dogs fed with them. From the late 1960's, when interest in this area was given a new boost along immunological lines, newer and more sensitive tools were used. Thus, Warshaw et al (1974) fed rabbits with tritiated bovine serum albumin (BSA), and found that about 1% was recovered in both lymph and blood in macromolecular form; this was identified as the original protein by gel filtration and immunodiffusion. This immunological technique was also used to quantitate the amount of cold BSA (Worthington et al (1974) and cold human serum albumin (HSA) (Andre et al 1974) absorbed in normal rats, and also the amount of cold HSA transported in an in vitro system (Brandtzaeg and Tolo 1977). The rates of absorption were generally low e.g. Andre et al (1974) observed that the

maximal rate of absorption of HSA in the serum (1 ml) at 2 hours
was about 0.3%.

The use of iodinated antigens and the choice of albumin as an
antigen, and the rat as a model (see Chapter 2) have been very
popular for in vivo absorption studies (Andre 1974; Walker and
Bloch 1977; Thomas and Parrott[*]; Parkins et al 1960). While
Hemmings and Williams (1978) recently reported that about 40% of
I^{125} proteins (rat IgG, bovine IgG and the gliadins) was
absorbed in antigenic form in the rat (about 5% in the
circulation, and the bulk in tissues), most other investigators
detected little or no absorbed antigen. Thus, Sanford and Noyes
(1958) found no absorption of Cr^{51} endotoxin in either the
tissue or blood of both normal and shock-induced dogs. No protein
bound radioactivity was detected in the circulation of rats fed
with I^{131} HSA (Parkins et al 1960) or mice fed with I^{125}
latexes (LeFevre et al 1977), while Thomas and Parrott (1974)
noted an extremely low absorption of protein-bound radioactivity
in the circulation of rats fed with I^{125} BSA.

The difficulties encountered in absorption studies in the intact
animal in terms of the poor recovery of the absorbed antigen, can
be diminished by using in vitro models. The most popular form is
the everted gut sac, originally devised by Wilson and Wiseman
(1954). Using this model, Walker et al (1972a) demonstrated
transport of I^{125} HRP and I^{125} BSA into the serosal sac in
very small amounts (0.5 -2 pmole/mg mucosa was absorbed from a 10
µM solution after 1 hour). Similarly, Nolan et al (1977) using
Cr^{51} endotoxin, showed that about 0.4% was absorbed from a 1
mg/ml bath solution in 2 hours, and that the absorbed material was

[*] 1974

still toxic and immunogenic. Another in vitro model was used by
Brandtzaeg and Tolo (1977) consisting of an intestinal membrane
set up in a diffusion chamber; using normal rabbit mucosa, 0.0032%
of HSA and 0.0016% of transferrin were absorbed in 2 hours.

The intestinal absorption of macromolecules has also been
followed by the systemic effects resulting from the absorption of
biologically active substances like hormones, kinins, toxins and
antibiotics. As early as 1923, Mills et al found that tissue
fibrinogen given orally to man could decrease the clotting time of
blood. Danforth and Moore (1959) showed that if rats were fed
insulin (with diisopropylfluorophosphate which inhibits insulin
degradation in the gut), a concommitant depression of blood
glucose ensued; the insulin absorbed from an everted gut sac model
was similarly found to be active. The enzymic activity of HRP was
exploited by Cornell et al (1971) while the sensitive limulus
lysate test was used to demonstrate the presence of endotoxin in
the portal blood of normal people (Jacob et al 1977). The extreme
toxicity of endotoxin particularly in conjunction with lead
acetate (Nolan et al 1977) or Actinomycin D (Shute 1977) in rats
or mice has also been used.

The systemic immunological effects resulting from oral feedings
are also taken as indirect evidence for macromolecular absorption.
These effects are manifested by an antibody response to the
antigen, by an anaphylactic reaction to it, or at the other
extreme, by tolerance. (The effects of antibody production and
tolerance will be considered in 1.6). The demonstration of
systemic hypersensitivity to ingested food goes back to the
1900's. Thus when normal subjects sensitized with the serum of

people hypersensitive to some protein, ate the same protein, they developed allergy in practically all cases (Brunner and Walzer 1928). More recently, Berstein and Ovary (1968) demonstrated passive cutaneous anaphylaxis in guinea pigs following the feeding of DNP-lysine (using rabbit anti-DNP injected intradermally) or egg-albumin (developed with rabbit anti-egg albumin). When schistosoma eggs were administered intragastrically or intraduodenally to adult mice, these mice developed systemic cellular hypersensitivity when challenged with the same eggs intravenously 2 weeks after the oral dose. (Perotto et al 1974).

1.4 The Gut and its Structure

The surface area of the small intestine in contact with the luminal contents is enormous not only by virtue of its length, but also by the presence of dense fingerlike projections or villi throughout its length. This is further increased 30-fold by the presence of microvilli on the free surface of cells lining the villi (the columnar epithelial cells, or sometimes known as enterocytes). There is a rapid turnover of these cells: the older cells at the tip of the villi are shed into the lumen to be replaced by crypt cells which migrate from the bottom of the villi towards the tip. In a normal person, the extrusion rate of old cells is similar to the mitotic rate for generating new cells, thus maintaining a constant villus length. However, if the mitotic rate is decreased, for instance by ionizing radiation, cytotoxic drugs or starvation, a flattening of the mucosa results and malabsorption ensues. On the other hand, if the mitotic rate is increased, as in coeliac disease, the villi become stunted and the crypts become elongated (see Bell et al 1976). Goblet cells

also originate in the crypts and extrude mucus to the lumen as they migrate upwards.

The microvilli are normally embedded in the mucus or glycocalyx (a polysaccharide-protein complex), and in this "brush border" area are found many of the digestive enzymes (disaccharidases, dipeptidases, phosphatases and dehydrogenases) and immunoglobulins.

The lymphoid cells of the gut may be divided into the effector blast cells found in the lamina propria and epithelium, and the affector cells found in the aggregated lymphoid tissues.

The majority of the blast cells in the lamina propria of many mammalian species, including man, secrete sIgA, while antibody-forming cells of the IgM, IgE classes are also found in smaller numbers (Baklien et al 1972). The intra-epithelial lymphocytes (Ferguson 1977) are interspersed among the columnar enterocytes in a 1:10 ratio (Fichtelius 1968), though the number of intra-epithelial lymphocytes may increase in certain diseased states, like coeliac disease (Bell et al 1976). The nature of these cells is not resolved; while Guy-Grand et al (1974) considered these to be T cells, Rudzik et al (1975a) found a mixture of T cells, B cells and "null" cells. Miller and Cudkowicz (1971) suggested these to be mast cells from their ultrastructure studies, and in line with this, Bienenstock (1974) was able to degranulate them with PHA or Con A, with subsequent release of histamine.

The gut-associated lymphoid tissues, comprising the Peyer's

patches, the appendix and the tonsil have been extensively reviewed because of current interest in their importance (Parrott 1976). The Peyer's patches (Peyer 1667) in particular, have important affector role (antigen sampling) in oral responses (Owen 1977). These are groups of subepithelial lymphoid follicles occuring throughout the small intestine, especially common in the distal ileum in mouse and man. The epithelium over these follicles is different from that in other areas of the mucosa (Owen and Jones 1974). Instead of columnar cells with their long microvilli, a specialized type of cell (the M cell) with short microfolds at its surface, is present; this is presumably an immature columnar cell (Owen and Jones 1974). There is also no glycocalyx over the microfold, fewer goblet cells (Page-Faulk 1971) and more intra-epithelial lymphocytes in this area. These lymphocytes lie adjacent to the short M cells, in the subepithelial zone, which also contains macrophages. Below this is the dome area, followed by the germinal centre of the Peyer's patch, and both are infiltrated with B lymphocytes.

The architecture of the gut is different in the germ-free animal (Abrams et al 1963) or in the intestinal isograft from fetal mice maintained in an antigen-free environment (Ferguson and Parrott 1972). In both cases, the epithelial cell turnover is slower than normal, and the number of intra-epithelial lymphocytes is much smaller. While the villi are tall and slender and the crypts are short in the germ-free gut, the villi in the antigen-free graft are short. Interestingly, despite no antigenic challenge in the latter, the intestinal graft is populated by both T and B cells, though in lower numbers. Furthermore, the Peyer's patches in the germ-free condition are much reduced in size and do not contain

germinal centres (Ferguson and Parrott 1973); both age and antigenic challenge influence the proportion of T and B cells here (Evans et al 1967) with a tendency towards the latter (B) type during maturation.

1.5   The Gut as a Digestive and Absorptive Organ

In a normal person, the absorption of food is virtually complete: 100% of carbohydrate, 95% of fat and about 90% of protein from a mixed meal are absorbed (see Bell et al 1976; Wiseman 1964; Gray and Cooper 1971).

Digestion begins in the mouth and stomach, and the acid chyme that enters the duodenum, is further digested by enzymes from the pancreatic juice and the succus entericus. Most of the protein is split into tri- and di-peptides where further breakdown to amino-acids occurs at the brush border or intracellularly. Similarly, disaccharides derived either from the amylytic action on carbohydrates, or directly from food, are split by brush border enzymes to the monosaccharides before absorption occurs. Fats are hydrolysed by lipases to fatty acids and monoglycerides, which are emulsified in bile to form micelles before being absorbed. Nucleic acids are presumably broken down to purine, pyrimidine, phosphoric acid and pentoses.

In man, the absorption of water and digested nutrients occurs mostly in the jejunum, while vitamin B12 and bile salts are actively absorbed in the ileum. Colonic absorption is confined to water and low molecular weight substances like glucose and inorganic acids.

1.6   The Gut as a Lymphoid Organ

It is currently believed that the Peyer's patch is an important sampling site of antigens derived from the gut, and it contains an enriched source of antigen-sensitive, IgA precursor cells. Though these cells do not produce antibody in situ following direct stimulation with BSA (Bienenstock and Dolezel 1971) or SRBC (Henry et al 1970), they are highly proliferative (Muller-Schoop and Good 1975; Clancy and Pucci 1978). From the work of numerous investigators (e.g. Craig and Cebra 1971; Rudzik et al 1975b; Gowans and Knight 1964; Guy-Grand et al 1974) the following picture of cellular traffic emerges: luminal antigen, absorbed through the M cells of the Peyer's patch, sensitizes the precursor cells (intra-epithelial lymphocytes?); these cells migrate to the mesenteric lymph node where they mature and pass into the thoracic duct, and then into circulation, before seeding selectively in the lamina propria. During this migration, there is also a progressive maturation (or enrichment) of cells containing IgA: from 2% in the Peyer's patch, to 50% in the lymph node, to 75% in the thoracic duct, and 90% in the lamina propria (Guy-Grand et al 1974). In support of the importance of the Peyer's patches as the site of precursor cells in the local humoral response, Robertson and Cebra (1976) showed that it was necessary to include a Peyer's patch in the rabbit ileal loops before specific antibody (sIgA) was produced to local stimulation with dinitrophenylated keyhole limpet haemocyanin or heat-killed Salmonella typhimurium. The many local responses observed with a variety of antigens, where sIgA is predominantly produced, (see Rowley 1974; Porter et al 1974; Ogra et al 1974) may thus be explained on the basis of

uptake of antigen by cells in the Peyer's patches, with subsequent sensitization, maturation and migration of these cells to the lamina propria, where they secrete sIgA. This may also explain the presence of specific sIgA in the colostrum of rabbits (Montgomery et al 1974) and humans (Goldblum et al 1975) from oral feeding of antigens. In agreement with this common mucosal system of antibody production, where cells sensitized at one mucosal site could subsequently seed at another (Bienenstock 1974; Rudzik et al 1975c), Ahlsteldt et al 1977 recently found that in women fed 10^9 E.coli cells, 0.1-1.0% of the cells recovered in their milk secreted specific IgA; since there was no serum response to this antigen, they suggested cell migration was responsible, rather than local stimulation at the mammary site by free, absorbed antigen. On the other hand, there are also reports demonstrating the presence of dietary antigens recovered in milk (Jakobsson and Lindberg 1978).

The concept above disregards the contribution of extra-intestinal tissues to the local response as antigen-sensitive sites. This may be true in situations where only local responses are observed (Ogra and Karzon 1969; Waldman et al 1968), but could nevertheless be over-presumptuous in others. This is because, not only can free antigen be recovered from the lymph and blood following an oral administration of the antigen (Warshaw et al 1974), but both mesenteric node and spleen can also develop specific antibody-forming cells, including those of IgA (Robertson and Cooper 1973; Bloom and Rowley 1979). Furthermore, both organs contain immunoblasts which can migrate to the gut mucosa (Parrott et al 1975a; Parrott et al 1975b).

It is equally overstating to claim that the local response is totally distinct from the systemic response resulting from the same oral challenge (though it is convenient to do so). As early as 1891, Ehrlich and in 1900, Uhlenhuth, observed precipitins in the serum following an oral challenge with proteins. Certainly, the relative contributions of the gut-associated lymphoid tissue, the mesenteric lymph node and the spleen to the development of a systemic response from an oral challenge is poorly defined. That the extra-intestinal lymphoid tissues may contribute very little to the systemic response was suggested by Heatley et al (1977), who found that both the primary and secondary systemic antibody responses to diphtheria toxoid and lauroylated HSA, when introduced intraluminally to rats, were not affected by previous splenectomy, mesenteric lymphadenectomy or by portacaval shunt; the antibody-forming cells were also found predominantly in the lamina propria, with only trace numbers in the subcapsular sinuses of the mesenteric lymph nodes in the case of the toxoid. Moreover, the sequential appearance of antibody forming cells following oral immunization in the lamina propria, the mesenteric lymph node and then, in the spleen of mice (Andre et al 1973; Rothberg et al 1973; Robertson and Cooper 1973) and the fact that no immunogenic level of antigen was detectable in the serum of rabbits despite a serum antibody response (Rothberg et al 1969) again suggested that the gut lymphoid tissue may be mainly responsible for any systemic response.

The question thus engendered is whether the many free antigens detected in the portal blood (Jacob et al 1977), in the systemic circulation and intestinal lymph (Warshaw et al 1974) after oral administration, are immunogenic. Some perhaps are, as indicated

by the greater serum antibody response in cirrhotic patients to bacterial antigens, compared with those of normal persons (Triger et al 1972); in these people, the Kupffer cell function and the liver is impaired (as discussed in 1.9). This observation appears to contradict that of Heatley et al (1977), in which their portacaval shunt (in effect, bypassing the liver trap) did not increase the systemic response to an oral dose. This discrepancy, and differences regarding the fate of immunogenic material, can perhaps be attributed to differences in the model system studied, and more importantly, in the type of antigen used. The physico-chemical nature of the antigen, including its degradability in vivo, undoubtedly determines the relative distributions of its immunogenic form in the intestinal tisues, in the portal vein, in the systemic circulation and in the intestinal lymph. Thus, Heatley and Stark (1975) found more unmodified HSA in the intestinal lymph of rats than the lauroylated form, because the latter had increased adhesiveness to cell surfaces, due to its hydrophobic nature. Similarly, while some (protein) antigens generated responses mainly in the lamina propria (Goldberg et al 1971; Crabbe et al 1969), particulate antigens may set up responses in the lymph node and spleen as well (Felsenfeld et al 1968; Bazin et al 1970). Thus, the filtration effect of the liver on the immunogenicity of absorbed antigens may only be obvious with particulate antigens. This is consistent with the findings of Thomas and Vaez-Zadeh (1974), that while little soluble antigen (BSA) was retained by the liver when given either by the mesenteric or femoral vein, significant amounts of a particulate antigen (S. adelaide) was retained, especially given by the mesenteric route.

An assessment of the quality of the serum response, in terms of the antibody class, avidity and functions (including immune elimination), may delineate the relative importance of the different lymphoid tissues contributing to the response. Thus, a predominant IgA serum response would implicate the gut-associated lymphoid tissue as an important source (particularly in the absence of responses in the node and spleen), and this has been observed in a number of instances (Heatley et al 1977; Crabbe et al 1969; Andre et al 1973; Bazin et al 1970). On the other hand, IgA may not be the predominant antibody class, as was observed with poliovaccine (Ogra et al 1968), and BSA and ferritin in conventional animals (Dolezel and Bienenstock 1971). Furthermore, Rothberg et al (1967) found no qualitative difference in the systemic responses between oral and parenteral immunization. Certainly, more comparative studies of such kind are desirable to answer the question on the fate and importance of immunogenic material orally absorbed.

A further point to consider, in deciding whether a systemic response develops from an oral immunization and the [*] of antibody formed, is the dosage of the antigen used. Conceivably, a very low dose would only stimulate the gut-associated tissue, generating virtually a local IgA response; whereas, a high dose could "spillover" into the circulation, and set up a serum response, not necessarily of the IgA type.

Any attempt to account for the systemic response resulting from an oral immunization, must also consider the development of systemic tolerance that is sometimes induced orally. The literature dealing with on orally induced tolerance at the mucosal

[*] class

level is scarce; Thomas and Parrott (1974) detected no intestinal antibody or antibody-forming cell in rats fed 25mg BSA daily for 4 days, and Strauss and Bienenstock (1975) observed mucosal tolerance in guinea-pigs fed with dinitrochlorobenzene (DNCB). In contrast, there are numerous reports demonstrating systemic tolerance from oral feeding of antigens. Chase (1946) discovered that repeated feeding of DNCB to adult guinea-pigs caused unresponsiveness to subsequent systemic challenge, as shown by the absence of an antibody response or hypersensitivity. Cantor and Dumont (1967) obtained similar findings with DNCB in dogs, and further found that this effect was abolished in dogs with a portacaval shunt. Together with the findings of Battisto and Miller (1962) that tolerance could be achieved by injecting the hapten or protein antigen directly into the mesenteric vein, it was postulated that while the particulate (immunogenic) part of the absorbed material was retained by the liver, the soluble portion escaped this filtration and tolerized the animal; however if the particulate matter was absorbed into the lymph, as in animals with portacaval shunt, then antibody response resulted. Thomas and Parrott (1974) demonstrated what they considered to be a low-zone tolerance in rats fed large amounts of BSA; whether tolerance resulted from the low, hence tolerizing dose of antigen absorbed or from the removal of aggregated material by the liver was not elucidated. Similarly, Hanson et al (1977) produced long-lasting specific immune unresponsiveness in mice fed a single dose (20mg) of ovalbumin or haemocyanin, but they could not produce this effect with a wide dose range of deaggregated ovalbumin administered systemically. Another example of orally induced tolerance was observed by Andre et al (1976) who made mice tolerant with intragastric doses of SRBC; IgA-antigen complexes

were advanced as the active tolerogenic factor. More recently, specific immune hyporesponsiveness in rats was observed after daily oral feedings of human serum albumin (Sewell et al 1979); immune-complexes were suspected to be responsible. Ngan and Kind (1978) concluded that suppressor T cells in the Peyer's patches were responsible for the tolerance observed in mice given ovalbumin orally; these cells also caused suppression of the antibody response in normal mice when given passively.

The importance of the gut as a lymphoid organ is also shown by the many forms of intestinal hypersensitivity (Ferguson 1976). Thus when parts of the small or large intestine of mice were transplanted into incompatible hosts, rejection occurred after a few days (Holmes et al 1971; MacDonald and Ferguson 1976). Arthus-type reaction was also produced in the colon of actively immunized rabbits with 1mg egg albumin given intramucosally (Goldgraber and Kirsner 1959). Further observations on intestinal hypersensitivity are discussed in 1.8.1.

1.7   Characteristics of Macromolecular Absorption in the Adult

1.7.1   The site and route of absorption

It is generally believed that absorption of antigens occurs in the small intestine. However, increased absorption of macromolecules from the colon is implicated from findings in patients with inflammatory bowel disease (see 1.10.2). Recently, Warshaw et al (1978) showed that the rat colon also absorbed H^3 BSA, though the amount was 10 times less than that absorbed by the

small intestine. In the rat, the jejunum and the ileum appear to
absorb equally well, as observed in the everted gut sac model
(Walker et al 1972a; Nolan et al 1977). The in vivo study of
Parkins et al (1960) in rats using I^{125}-HSA, however suggested
the distal ileum as the main region of absorption. In mice, the
Peyer's patches in the distal ileum were found to be more active
in taking up Salmonella organisms (Carter and Collins 1974) and
HRP (Owen 1977) than those in the proximal intestine.

Early microscopic studies using HRP showed that this antigen was
taken up and transported by columnar cells, especially the more
matured cells near the distal end of the villi (Cornell et al
1971), and also intercellularly (Walker and Isselbacher 1974;
Rhodes and Karnovsky 1971). However, in these studies, the
Peyer's patches were not looked at. Recent studies have
implicated these to be important sites of antigen uptake. Thus,
Owen (1977) observed specific uptake of HRP by the M cells of the
Peyer's patches in mice, whereas there was no uptake by the
columnar cells of the villi or the lymphoid follicles. Similarly,
LeFevre et al (1978) found an accumulation of latex particles in
the Peyer's patches of mice when these particles were given in the
drinking water for 2 months. Earlier studies had also shown
specific uptake by the Peyer's patches, including ferritin in mice
(Bockman and Cooper 1973), trypan blue in dogs and rabbits (Kagan
1931) and Indian ink and carmine in rabbits (Kumagai 1922).

There is little information on whether the absorbed antigen
enters the intestinal lymph or the portal blood. This may well
depend on the nature of the antigen (see 1.6). Antigens have been
found in both lymph (Heatley and Stark 1975) and portal blood

(Jacob et al 1977) while Warshaw et al (1974) recovered 1% non-dialysable label from both lymph and portal blood of rats fed with H^3 BSA. Further, the relative importance of the intercellular transport of antigens to intracellular machanisms (see next section) remains to be resolved.

1.7.2   The kinetics and mechanism of absorption

Following oral feeding, the absorption of H^3-BSA into the intestinal lymph of rats was rapid, peaking at 60 minutes, and this gradually fell off, such that at 3 hours, very little label could be recovered (Warshaw et al 1974). In the same study, the antigen was increasingly absorbed into portal blood between 30 and 90 min, after which very little increase was noticed. Furthermore, the amounts absorbed into both lymph and portal blood was linearly proportional to the dose given, for the range 0.5mg/kg body weight - 500mg/kg; however, the data also suggested a saturation effect from the highest dose used. Saturation kinetics were indeed observed by Nolan et al (1977) using everted gut sacs with Cr^{51} endotoxin (0.05mg/ml - 2.0mg/ml bath concentration), from which they suggested a carrier transport system for macromolecules (like those for amino-acids and peptides). On the other hand, pinocytosis followed by vesicular transport has been advanced in both the columnar cells (Cornell et al 1971) and the M cells (Owen 1977). In the former, the antigen is endocytosed in-between the microvilli after adsorption, and is then transported in vesicles which coalesce with lysosomes, in a manner analogous to neonatal transport (Walker et al 1972b). In the latter, it is postulated that there is tubular uptake and vesicular transport of the antigen in the M cell without any

fusion with lysosomes; the antigen is released into the extracellular space, which is then taken up by the intra-epithelial lymphocytes. These cells presumably then migrate.

While some workers (Ravin et al 1960; Gans and Matsumoto 1974) believed the intestinal absorption of macromolecules to be a passive process (partly because of the small amount of antigen absorbed), an active mechanism is generally favoured, since saturation kinetics were observed (Nolan et al 1977), and the absorption could be inhibited by 2,4-dinitrophenol, a metabolic inhibitor (Walker et al 1972b).

1.8   Factors Affecting Macromolecular Absorption

1.8.1   Immunological

The discovery of secretory IgA (Heremans et al 1959) and the demonstration of its predominance in secretions (Tomasi et al 1965) have prompted intensive investigations as to its functions, many of which still elude us. It is believed to lack secondary functions such as opsonization (Steele et al 1974) and complement-mediated activities (Ishizaka et al 1966; Eddie et al 1971). While the neutralization of virus (Dowdle et al 1971) and possibly toxin (see Tomasi 1976) has been described for sIgA, its role in controlling bacterial colonization, presumably by preventing adherence to the mucosa, has only recently been proposed (Rowley 1974; Williams and Gibbons 1972). Another possibly important role for sIgA is in blocking the uptake of

harmful macromolecules by the body. This stems from the observation that people with IgA deficiency often have elevated levels of circulating antibodies to dietary antigens (Buckley and Dees 1969); moreover, Taylor et al (1973) noted that infants at 3 months of age, of allergic parents showing a transient serum IgA deficiency, developed infantile eczema and positive skin test to common antigens within the first year. However, direct demonstration of the blocking function of IgA is limited. An IgA myeloma (MOPC - 315, with specificity against the dinitrophenyl group, DNP) was shown to block the absorption of I^{125} - HSA into the circulation of rats from the trachea (Stokes et al 1975). In another study, Andre et al (1974) observed a reduced uptake of HSA (as measured in the mesenteric venous blood) in rats that were immunized with a single intragastric dose of HSA (200 mgs) two weeks previously. The reduction was 33%, 25% and 60% of that in unprimed animals at 1, 2 and 3 hours respectively after the challenge dose. The blocking activity was related to the immune intestinal secretions, as they also caused a decrease in antigen uptake when given passively with the antigen. However, in this study, the antibody class involved was not elucidated but presumed to be IgA. In contrast, Bockman and Winborn (1966) found increased uptake of ferritin in the enteric surface coat and within the intestinal mucosa of hamsters sensitized with ferritin intraperitoneally or subcutaneously. However, in this microscopic study, the amount of ferritin absorbed into circulation was not determined; this could be different from the apparent enhancement observed at the mucosal level due to blocking antibodies.

It should be pointed out that there are other immunoglobulin classes besides sIgA in the intestinal secretions. Thus, IgM

(mostly associated with secretory components like IgA), IgG and IgE are found at low levels (Clancy and Bienenstock 1976); the low levels could reflect greater degradation in the lumen (Horsfall et al 1978) due to lesser stability. While IgM may be preferentially transported (Brandtzaeg 1975), IgG is believed to be passively derived from the serum (Heddle and Rowley 1978). The source of IgE in secretions is not known; IgG1, was found to be the antibody induced orally in rat secretions, and this was responsible for blocking the uptake of the specific antigens, HRP (50 -70% inhibition) or I^{125}-BSA (30 - 50%) in everted gut sac models (Walker et al 1972a). This inhibition was also observed in rats immunized intraperitoneally five times with the antigens, though it was less consistent compared with rats orally immunized; it was inferred that the production of another Ig class, IgGa, together with IgG1, was responsible for the inferior inhibition (Walker et al 1973). Further studies by this group suggested the mechanism of inhibition was one of immune exclusion rather than of immune elimination. Thus, immune-complexes were recovered from the mucus of immunized animals, and the antigen in these complexes was more rapidly degraded by pancreatic enzymes than the free antigen (Walker et al 1975).

There are no comparative data on the efficiencies of sIgA and the other Ig classes in blocking the uptake of macromolecules. From the many biological functions known of these classes (Steele et al 1974) including their relative binding efficiencies, it would appear that IgM and IgG could do equally well, if not better than sIgA in this respect; in other words, the teleological reasons for the existence and predominance of sIgA in secretions is still a mystery. However, it is possible that the biological

inertness of sIgA may really be advantageous, especially in an environment constantly loaded with large amounts of antigens. This is apparent from the many reports indicating deleterious effects resulting from the combination of antigen with the other Ig classes in the gut, while hypersensitivity reactions due to sIgA have not been observed (Ferguson 1976). Thus, Brandtzaeg & Tolo (1977) using an in vitro model, found that while IgG1 blocked the specific absorption of HSA across rabbit mucosa, this reaction enhanced the transport of an unrelated protein, transferrin. Furthermore, Bellamy and Nielsen (1974) observed the emigration of large numbers of neutrophils into the intestinal lumen of actively sensitized pigs, or in pigs passively sensitized with immune sera given intravenously, when challenged intraluminally with the specific antigen, BSA. Intestinal anaphylaxis was also seen in guinea-pigs sensitized by an intraperitoneal injection of horse serum 3 - 12 weeks before an oral challenge of horse serum (Hettwer and Kriz 1925); this was also seen in man passively sensitized with atopic sera (containing reaginic antibodies to peanut) at the mucosae, when 10gm of peanuts was ingested 1-3 days later (Gray et al 1940). Arthus-type reaction due to immune-complexes containing IgG and complement has been implicated in Crohn's disease (Monteiro et al 1970; Green and Fox 1975) and in experimental granuloma (Germuth and Pollack 1967). Thus IgM, IgE and particularly IgG antibodies can mediate inflammatory, hypersensitive reactions in the gut when complexed with their specific antigens; the gut may thus be regarded as being in a state of chronic inflammation under continuous antigenic challenge (Clancy and Bienenstock 1976). This may result in the pathotopic potentiation of mucosal surfaces (Fazekas de St.Groth and Donnelly 1950), whereby serum-borne immunity is predisposed to the mucosal

environment. The role of sIgA may not merely be to block antigen uptake, in which it has not been shown to be highly effective so far, but also to modulate the biological effects of the other Ig classes (Brandtzaeg and Baklien 1976), and the immune response (both local and systemic) to antigens presented orally. Thus, IgA could reduce the antigenicity or immunogenicity of particles (Brandtzaeg et al 1968; Eddie et al 1971), block the release of histamine by IgE in nasal secretions (Turk et al 1970), and block bacterial lysis (Hall et al 1971; Griffiss 1975).

The role of cell-mediated reactions in influencing the absorption of macromolecules is obscure. Delayed hypersensitivity has been observed with DNCB in guinea-pig and pig colon (Bicks et al 1967) and in rats infected with Nippostrongylus brasiliensis (Ferguson and Jarrett 1975). Understandably, such reactions may inhibit the uptake of specific antigens, while at the same time the absorption of other unrelated antigens may be increased.

1.8.2   Others

The intestinal absorption of macromolecules can also be influenced by many physico-chemical and physiological factors, which are only briefly discussed here.

It is expected that intraluminal factors, like disturbances of motility and gastric or pancreatic insufficiency, will affect the effective antigenic dose available for absorption. Thus, neutralization of gastric acidity with bicarbonate resulted in better immunization with live bacteria (Bloom 1979) and toxoid (Pierce et al 1977; Holmgren et al 1975), while pancreatic

ligation enhanced the absorption of insulin in rats (Danforth and Moore 1959) or decreased the degradation of immune-complexes at mucosal surfaces (Walker et al 1974b). Also, cystic fibrosis in man has been found to be associated with allergy and the presence of immune-complexes and anti-dietary antibodies (Allan et al 1975; McFarlane et al 1975; Wallwork et al 1974). Danforth and Moore (1959) found it necessary to use a trypsin inhibitor (diisopropyl fluorophosphate) in order to demonstrate the absoprtion of insulin. There are many factors that can affect the physical integrity of the mucosal barrier, and these consequently influence the absorption of antigens. Thus, shock, trauma, diseases of the tract (ulcerative colitis, Crohn's disease, coeliac disease, gastrointestinal allergy and inflammatory diarrhoea), immunosuppressive drugs, and toxic substances like endotoxin (Walker and Porvaznik 1978) could cause greater penetration of macromolecules. The mucus is an important component of the mucosal barrier. While small molecules like ions, amino-acids and sugars diffuse freely through it, macromolecules are excluded, according to their molecular weight and surface structure; those with similar composition to the mucus-glycoproteins would be more miscible with the mucous phase (Edwards 1978). Further, IgA molecules, with a mucus-like glycoprotein sequence at the hinge region between the Fc and Fab regions (Kornfeld and Kornfeld 1976) are thought to sit in the interface between the mucus and the overlying fluid, with its mucus-like portion in the mucus and the rest in the fluid, thus forming a monolayer on the surface of the mucus. Hence, the mucus, with its complement of IgA molecules may protect the underlying mucosal cells from macromolecules and microorganisms in the lumen. It is reported that the mucous layer is absent over the M cells of the Peyer's patches, thus allowing

direct contact between the luminal antigens and the M cells (Owen 1977). This may account for the accumulation of antigens (LeFevre et al 1978) and bacteria (Carter and Collins 1974; Hohmann et al 1978) in these follicles. Other bacteria, like V.cholerae could attack the mucus with their hydrolytic enzymes like neuraminadase, and thus, may not show any predilection for the Peyer's patches. Other agents that destroy the mucus, like dithiothreitol, could also enhance macromolecular absorption (Walker et al 1974a).

Changes in the rate of macromolecular absorption may also be brought about by modifying the electrostatic nature of both the antigen (Heatley and Stark 1975) and the cellular membrane. The latter can be mediated by charged polypeptides; for instance, Smith and Burton (1972) found increased uptake of albumin and globulin in gut sacs from neonatal pig when polyornithine was used.

Finally, (as discussed by Walker and Isselbacher 1974), factors affecting lysosomal stability, i.e. intracellular digestion, also affect the amount of antigen transported. While Vitamin A (see Falchuk et al 1978), streptolysins, radiation and endotoxins reportedly increase the lability of lysosomes, steroids apparently inhibit lysosomal function and hence could cause increased macromolecular absorption.

1.9 The Liver as a Further Barrier to Absorbed Macromolecules

The liver, developed from the fetal gut, may be regarded as a second filter to the absorption of macromolecules from the intestine; it thus prevents any antigen that escapes the first

barrier (the gut) from spilling over to the systemic circulation and causing injury to the other organs. This function (of its Kupffer cells) is evidenced by the presence of endotoxaemia in the portal blood, but not in the systemic circulation, of patients with normal liver function (Prytz et al 1976; Jacob et al 1977). A direct role of the liver in detoxifying endotoxin comes from numerous studies (Farrar and Cormin 1966; Rutenberg et al 1967; Rippe et al 1974), while on the other hand, a direct hepatoxic effect of endotoxins, especially on a previously injured liver, is also known (Nolan 1975; Grun and Liehr 1977; Ali and Nolan 1967).

The liver may not trap soluble antigens as well as particulate antigen; but if they are suitably opsonized, the retention is good, especially when given by the mesenteric route. Thus, Thomas and Vaez-Zadeh (1974) found little retention of BSA (about 5%) by the liver of rats when given by either the femoral or mesenteric vein. However, when it was complexed with an equimolar amount of specific antibody, the retention was 55% and 85% of the dose by the respective routes. Immune-complexes formed at antigen excess were*retained. Similar findings were made with a particulate antigen, formalinized S.adelaide, when given to immune animals; unlike BSA, this antigen was also significantly retained by the normal liver when given alone (20% by the mesenteric route and 15% by the femoral route).

There are numerous indirect observations supporting the importance of the liver as a filter to absorbed antigens, and consequently, as a regulator of the immune response; it is generally believed that it only degrades antigen trapped by it and does not process it for priming (Franzl 1972). These observations

*less efficiently

are made experimentally or clinically, in situations where the liver filtering function is depressed (as in cirrhosis, or by blockade with carbon, esters, endotoxin and immune-complexes) or excluded (as by portacaval shunt and superior-mesenteric artery occlusion). The assessment is usually based on changes in the immune response, including the induction of tolerance; on the presence of immune-complexes or their sequelae; and on the extra-hepatic symptoms of endotoxaemia (Nolan 1975; Wilkinson 1977; Bradfield 1974). Thus, patients with chronic liver disease were found to develop high levels of antibodies to enteric E.coli and bacteriodes but not to non-enteric organisms like H.influenza (Triger et al 1972). Elevated anti- E.coli titres and immunoglobulin levels were also observed after portacaval shunts (Bjornebone et al 1972; Keraan et al 1974). Similarly, Triger et al (1973) found lower antibody titres to SRBC in rats when the antigen was given by the portal vein than when given by the inferior vena cava. Finally, there are many clinical findings associating cirrhosis with endotoxaemia, due to endotoxins absorbed from the gut (Caridis et al 1972; Shute et al 1977; Woodruff et al 1973) and the effects of endotoxaemia, including functional renal failure (Clemente et al 1977), haemorrhagic gastritis (Shute et al 1977), and intravascular coagulation (Wilkinson et al 1974).

1.10   Diseases Related to Macromolecular Absorption

The absorption of small amounts of macromolecules from the gut may be a normal event (Jacob et al 1977), without any evident ill-effects in a normal person. However, a defect in the first barrier (the gut, as discussed in 1.8) or in the second (the

liver, as discussed in 1.9) could result in a greater uptake of macromolecules, and this may result in disease, localized either intestinally or extra-intestinally. Thus, children who had acute gastroenteritis were later found to develop hypersensitivity to cow's milk (Harrison et al 1976). Since IgA reportedly inhibits macromolecular uptake, a selective deficiency in this immunoglobulin class could mean a tendency towards gut-associated diseases, immune-complex disorders and atopy. The evidence for this is however, not convincing, as many people with selective IgA deficiency are apparently normal. In fact, IgA deficiency is more common than deficiencies in other Ig classes, occurring at a rate of 1 in 500-700 individuals (Bachman 1965). There are nevertheless, cases of IgA deficiency associated with coeliac disease (Savilaht et al 1971), autoimmune diseases (see Ammann and Hong 1971), liver cirrhosis (Wilson et al 1968) and systemic allergy (Kaufman and Hobbs 1970). Moreover, infants with transient IgA deficiency at 3 months were found to develop atopic eczema within the first year (Taylor et al 1973).

Some diseased states, on the other hand, may result from an abnormal immune response to a normal uptake of antigen (as in coeliac disease?), rather than to an abnormally high uptake of antigen itself. In still some others, both the rate of antigen uptake and the immune response may be normal, but the absorbed macromolecule is toxic (as with bacterial exotoxins and viruses). As such, while the former group may show linkage with the histocompatibility genes (presumably linked to immune-response genes), the latter may not.

1.10.1   Extra-intestinal

An important extra-intestinal manifestation of macromolecular uptake from the intestine is liver cirrhosis. Experimentally, this can be induced in rats fed on a choline-deficient diet, but the intestinal flora must be present (Salmon and Newberne 1962), as removal of the flora with neomycin (Rutenberg et al 1957) or the use of germ-free animals (Luckey et al 1954) prevented such a development. This suggested that endotoxin absorption from the gut was responsible for the induction; this is further supported by the observation that purified endotoxin could induce cirrhosis in these animals even when neomycin was used (Broitman et al 1964).

In those people with liver dysfunction (arising from cirrhosis, hepatitis or other blockading causes), ordinarily innocuous amounts of absorbed antigen become toxic when spilled over to the circulation. In the case of endotoxin, these extra-hepatic manifestations include pyrogenic reaction, functional renal failure, coagulation disorders and gastric mucosal haemorrhage, as already discussed in 1.9.

Certain soluble antigens, e.g. BSA, are not readily sequestered by the liver, even if complexed with preformed antibody especially in disproportionate ratios (in antigen excess, for instance) (Thomas and Vaez-Zadeh 1974), or if the antibody is of low avidity (Bradfield 1974; Soothill 1977) or of a particular class. IgA is not opsonic (Steele et al 1974), and presumably also low in affinity. This may explain the common occurrence of IgA-related glomerulonephritis (Morel-Moroger et al 1972), particularly if the

antigen (dietary or viral) is absorbed through mucosal surfaces and IgA is consequently produced. Conceivably, these manifestations can be mimicked by any Ig classes so long as the complexes formed are not cleared effectively by the liver, either for the reasons mentioned above, or as in cases of complement insufficiency (Moncada et al 1972; Peters and Lachman 1974; Ehlenberger and Nussenzweig 1977).

A less understood area in which the absorption of food macromolecules can have extra-intestinal implications is psychiatric illness (Dohan 1978; Mackerness 1976); certain foods are known to affect the mood and behaviour of some people (Finn and Cohen 1978) including the aphrodisiac types known since biblical times!

1.10.2 Intestinal

The deleterious effects of macromolecular absorption as manifested in the gut are extensively reviewed (Wright 1977; Walker and Isselbacher 1974; Brandtzaeg and Baklien 1976); these are briefly considered here. In many of these, the pathogenesis and the aetiology are not known, while reports on these are often conflicting. An increased absorption of gut antigens (except in Crohn's disease; Taylor et al 1964) - indicated by increased antibody titres to them - and extra-intestinal manifestations of the disease in the form of immune-complex and atopic disorders, are commonly noted. The role of cell-mediated immunity in these diseases remains to be defined. Recently, Ferguson and MacDonald (1977) suggested that a cell-mediated reaction to food, microbial, parasite or other antigens, mediated by lymphokines, could be

responsible for many of the conditions associated with villous atrophy and crypt hyperplasia, resulting in changes in enterocyte turnover rate and malabsorption.

1.10.2.1   Coeliac disease

This is believed to result from an immunological reaction in the jejunum to ingested gluten (Booth 1970); relief is obtained when gluten is removed from the diet. Certain histocompatibility groups are more prone to it, namely, HLA-B3, HLA-B8 and HLA-DW3 (Keuning et al 1976), which suggests an abnormal immune response in these people. However, while a decreased serum response in these patients was recorded in one finding to an oral challenge with poliovaccine (Beal et al 1971), an increased serum response (that was predominantly IgA) was observed by Mawhinney and Love (1974) in another group, given the same treatment. Similar conflicting results were obtained with tetanus toxoid (Beal et al 1971; Pettingale et al 1970). Immune-complexes were observed following challenge with gluten, in both serum and intestine; in the latter, IgM and complement were seen (Booth et al 1977). In the serum of untreated patients, C2, C4 and IgM levels are generally decreased, while that of IgA may be increased. Anti-reticulin antibodies are present in over 50% of adults and virtually in all children. Thus, the reaction between gluten and a complement-fixing antibody (IgM or IgG) could cause cell damage and disease.

1.10.2.2   Gastrointestinal allergy

Allergy to many types of food, notably milk, eggs, fish and

chocolate (Bleumink 1970), either immediate or delayed, and either intestinal or extra-intestinal, is common; it should however be distinguished from the non-immunological causes of food intolerance (e.g. enzymic deficiency and non-specific release of histamine).The best documented form is cow's milk allergy in the infant; the immunological basis is varied and has been found to be an Arthus-type, delayed hypersensitivity or immediate hypersensitivity (type I) even in the same children (Shiner et al 1975); support for type I hypersensitivity comes from the relief obtained with disodium cromoglycate which abrogated the Prausnitz-Kustner reaction to beta-lactoglobulin in infants intolerant to milk (Freier and Berger 1973). The extra-intestinal manifestations of gastrointestinal allergy are many; these include eczema, asthma, urticaria and migraine, which also commonly occur in the same children showing cow's milk allergy (Goldman et al 1963).

1.10.2.3   Inflammatory bowel disease

This group of disease includes two types: ulcerative colitis (Wilks and Moxon 1875) and Crohn's disease (Crohn 1932). Whether they stem from the same aetiology (which is still unknown) is uncertain. The former is a chronic inflammatory disease of the colon, maximal at the rectum and confined to the mucosa, while Crohn's disease is characterized by the thickening of the bowel wall with deep ulceration, fissures and fistula formation (Wright 1977). The extra-intestinal complications of both include atopic disorders, skin rashes, arthritis and liver diseases, probably resulting from the deposition of immune-complexes from gut-derived antigens. Immediate hypersensitivity (type I) to milk was

suggested in some cases of ulcerative colitis. This was based on the increased IgE cells in the mucosa; the relief obtained with disodium cromoglycate and from abstaining milk; and further, on the association with atopic disorders. Autoimmunity was considered in others, where cross-reactivity was shown between enterobacterial antigens (e.g. E.coli 014) and the colonic epithelium (Asherson and Holborow 1966; Perlmann et al 1967). Another theory comes from the observation that lymphocytes recovered from patients with inflammatory bowel disease were cytotoxic for normal colonic cells (Perlmann and Broberger 1963). Further, normal lymphocytes could be made cytotoxic by incubation with the serum of these patients (Shorter et al 1973; Stobo et al 1976). However, while this array of immunological abnormalities (reviewed by Jewell and Hodgson 1976) requires further investigation, they may really be just epiphenomena, secondary to an infective aetiology. The possibility that a virus or a bacterium (e.g. mycobacterium) could be responsible was recently suggested by the ability to culture it (Aronson et al 1975; Burnham and Lennard-Jones 1978; Gitnick et al 1976) and the ability to infect and produce granulomata in animals with extracts of the diseased colon (Mitchell and Rees 1970; Cave et al 1975). Furthermore, the familial incidence of cold lymphocytotoxic antibodies (Korsmeyer et al 1975) and the disease (Singer et al 1971) is also consistent with a common environmental aetiology.

1.10.2.4   Toxigenic diarrhoea

Many enteropathogens cause diarrhoea through their exotoxins, including V.cholerae and E.coli (Harris 1976). The V.cholerae toxin has been extensively studied, and it has been postulated

that it acts by stimulating adenyl cyclase activity, which in turn causes an accumulation of cyclic AMP in the cell (Van Heyningen et al 1976). Since this enzyme exists primarily on the basal-lateral membranes (Parkinson et al 1972), uptake of the toxin into the mucosal cell is necessary. There is evidence for this (Kao et al 1972; Van Heyningen and King 1975; Bennet et al 1975) and recently, Strombeck (1973) found that colchicine, which inhibits macromolecular transport in the microtubules of cells (Dustin 1978), prevented the toxin from acting even when it was absorbed on the brush border membrane. The mechanism of action of other bacterial exotoxins may be different (Keusch et al 1972) but cellular uptake may still be a necessary event.

1.11   The Importance of Studying Macromolecular Absorption

From birth, the gut is challenged constantly with a heavy and varied load of antigens; it soon becomes colonised with a microflora, whose released products add to the antigenic load. This challenge from the microflora and dietary factors not only moulds the architecture of the gut, but also primes its immunological apparatus, and contributes to the defence of the gut (Bryant 1972). How these are done remains speculative. Further, how the microflora successfully establishes itself in the gut is a mystery, since immunological mechanisms are operative here to exclude the "non-self". Perhaps, both microflora and some dietary factors share antigens with the gastrointestinal tissues, and this may be responsible for some autoimmune states (e.g. Crohn's disease?).

These are only a few of the many questions on the gut that

demand serious investigation. The immunological importance of the gut was only recently acknowledged, and the interfaces between immunology, gastroenterology, biochemistry, physiology and electronmicroscopy in this area certainly represent exciting new frontiers in research (Medical Research Council, London 1976; Work Group VII 1975). Specifically, the area of intestinal absorption of macromolecules is still new and there is a dearth of knowledge in it pertaining to basic immunology, and the many diseases related to it (Lancet 1978). The aims of this thesis are to try to answer some of these uncertainties.

There is convincing evidence from a number of different investigators, using a number of different systems, that macromolecules are absorbed from the intestine. However, the amount absorbed is disputed; though most agree this amount is small and nutritionally unimportant, extremes are also found - from no absorption (Sanford & Noyes 1958) to as much as 40% (Hemmings and Williams 1978). Studies are thus required to settle this question: are the differences due to the different systems (including animals) and antigens used? Or are they caused by the different assay techniques? This latter possibility is important, since difficulties encountered in these studies are enormous (see Chapter 2) and further, Andre et al (1974) obtained wide discrepancy in the amount of HSA absorbed as measured by radioimmunoassay and immunodiffusion. It is likely that the bulk of antigen absorbed in the intact animal ends up in the body tissues (viz. the intestine and liver), so that the amount recovered in the serum may be minimal (depending on the nature of the antigen). Yet many investigators have only considered the serum. More studies, with better models and techniques, are

required to study the fate of the absorbed material in the whole animal, where _in vitro_ models would be limited in this respect.

While the antigenicity of the absorbed material is usually confirmed with specific antibodies, its immunogenicity is rarely investigated. This determination is important in understanding the inductive mechanisms relevant to both local and systemic responses to oral immunization. How important are the Peyer's patches, the lymph node, the spleen and the liver in such responses? There is obviously a balance between the induction of immunity and tolerance to an oral antigen: what controls this fine balance? This may be different from systemic challenge, as the liver and the Peyer's patches are important controlling sites in the oral route. In the Peyer's patches, both antigen-sensitive cells and suppressor T cells are present; though these sensitized cells have been intensively studied, nothing is known about the antigen contained therein - is it "processed", and released? Some antigen is transported transmurally through the lamina propria and released into circulation or lymph : what role has it in the induction of serum responses, and what class does it stimulate?

Evidence for the role of sIgA in blocking antigen uptake is only circumstantial. The link between selective IgA deficiency and increased antigen uptake, or with atopy and other gut-associated disorders, is weak, and needs further clarification. The role and biological significance of other Ig classes in the gut (IgM, IgG and IgE) in preventing macromolecular absorption is little known, but evidence so far suggests that these antibodies, when complexed with their antigens, could mediate inflammatory reactions in the gut. This could be accompanied by increased permeability to other

antigens, deposition of immune-complexes in both serum and intestine, cell damage with consequent release of histamine, and also, emigration of neutrophils into the lumen. These immunological complications should be further looked into, as possible underlying causes (or as epiphenomena) of coeliac disease, gastrointestinal allergy and inflammatory bowel disease. Furthermore, the role of sIgA in controlling these reactions should be clarified since this knowledge could have therapeutic implications. If sIgA is found to be the privileged antibody to exclude antigens, then oral vaccines should be tailored to produce this immunoglobulin only - hence the route and fate of antigen uptake is important.

There are many diseases associated with the absorption of macromolecules (as discussed in 1.10). The pathogenesis and aetiology of many of these require further elucidation. The immunological observations are often conflicting; more clinical findings are necessary and perhaps, suitable animal models could be found. The role of cell-mediated immunity in these diseases is still not understood and study in this area could prove rewarding.

This wide spectrum of investigation is of course beyond the scope of this thesis, but attempts will be made to describe as quantitatively as possible the kinetics of absorption of two common types of gut-associated antigens and the effects of specific antibody on the absorption.

2.   CHAPTER 2. THE PROBLEMS IN ORAL ABSORPTION STUDIES

The difficulties encountered in oral absorption studies have been known since the late 19th century. This is evident from the many theories put forward on the form of protein absorbed (Chapter 1). The sensitivities of the techniques used were instrumental in formulating these ideas; thus, only with a better technique did Van Slyke and Meyer (1913) find absorbed amino-acids in the circulation, and this finding has since revolutionized thinking in this area. More recently, the question of whether macromolecules are absorbed from the adult intestine, and in what quantities, has posed similar problems.

2.1   What is an Absorbed Antigen : How and Where do We Look for

It?

Unlike studies concerned with the transplacental transport of macromolecules (Wild 1974), studies on the intestinal uptake of macromolecules in vivo are hampered by complexities such as the presence of digestive enzymes in the intestinal tract, and the enormous difficulties in recovering the absorbed material. The digested products in the gut consist of a spectrum of molecular sizes, ranging from the predominant building blocks (amino-acids, monosaccharides, etc.) to intact macromolecules; conceivably, all forms are absorbed, though at different rates. The macromolecules may be further digested intracellularly as they are transported through the intestinal mucosae and in the body tissues. The molecular heterogeneity may thus be greatest at the mucosae and

least in the circulation (where the smaller units predominate). Hence, the first question arises: what molecular sizes should be looked for in studies on the intestinal absorption of macromolecules? Nutritionists and physiologists have recently been concerned with the uptake of dipeptides, which are identified by biochemical techniques (Peters et al 1970). Immunologists are however, interested in oligomers which can be detected by various immunological means. Macromolecules as small as hexasaccharides or pentapeptides, with molecular weights less than 1000, can be efficiently detected by their inhibition of a standard immunoassay like precipitation (see Kabat 1976a); these "haptens" are recognised by preformed antibodies but cannot stimulate antibody production. Van Vunakis et al (1966) examined the inhibition of C^1 fixation of poly-D-lysine with specific antiserum by oligolysines. The inhibitory power (on a molar basis) increased sharply from the dipeptide to the pentapeptide, after which there was no further increase. Consequently since the amount of orally absorbed macromolecule is generally small, it may be very difficult to detect oligomers smaller than pentapeptides in inhibition assays. Berstein and Ovary (1968) elicited passive cutaneous anaphylaxis in guinea-pigs fed with egg albumin; presumably, in this reaction, the molecular size required is at least a tetrapeptide (Schlossman et al 1966). Larger, multivalent macromolecules (antigens) may require cross-linking by antibodies in order to be detected by direct precipitation, whether in solution or in gel, or with the help of secondary agents (e.g. antibody, ammonium sulphate or polyethylene glycol). The immunogenic form i.e. that which can stimulate a humoral or cellular immune response, must be at least as large as the antigenic form. Synthetic polypeptides with low molecular weights

of about 4-5,000 (see Sela 1966), and the nonapeptide, arginine vasopressin (see Butler and Beiser 1973), have been reported to stimulate antibody production in animals. The tolerogenic forms may be smaller than the immunogenic form (Cantor and Dumont 1967) or as big as immune-complexes (Andre et al 1976). It should be borne in mind that haptenic molecules absorbed into the circulation, may complex with host proteins (e.g. by transconjugation) and hence assume forms of greater biological significance. Such a possibility has been documented with penicillin (Levine 1965). Thus, depending on the kind of immunoassay used, macromolecular absorption from the gut may very considerably among investigators, even for the same protein, since these assays may detect different molecular forms and because of this and other reasons, have different sensitivities. The sensitivity of an assay particularly depends on the avidity of the antiserum used (Parker 1976; Tosi and Celada 1974), and this can sometimes be increased at the expense of the resolution or reliability of the system. Inhibition reactions in general are more sensitive than direct precipitin tests; however, while Andre et al (1974) found this to be the case, they also observed spurious results in their radioimmunoassay.

Many investigators (Warshaw et al 1974; Andre et al 1974; Swarbrick et al 1979) have only looked for absorbed material in the circulation, probably because this is the easiest source. However, it is likely that the absorbed antigen is rapidly removed to the tissues, as with amino-acids (Van Slyke and Meyer 1913) and dipeptides (Adibi et al 1977). It may therefore be important to measure absorption at these sites. The intestinal mucosa perhaps retains the most antigen, where further degradation (or

processing) may occur. Consequently, there are numerous microscopic studies demonstrating the deposition of antigen (tubercle bacilli, horseradish peroxidase, latexes, carbon particles and ferritin) in this tissue particularly in the Peyer's patches (Kumagai 1922; Owen 1977; LeFevre et al 1978; Joel et al 1978; Bockman and Winborn 1966) whereas it was difficult to demonstrate this in other tissues (Joel et al 1978). Quantitative studies are however difficult to perform in this area, since the problem of distinguishing between material that is merely adsorbed to the mucosal surface and that actually absorbed intracellularly exists. The liver, acting as a filter between the gut and the circulation, supposedly takes up much of the macromolecular material absorbed through the mucosae, especially the more particulate antigen (although Hemmings & Williams (1978) indicated the importance of skeletal tissues in this sequestration). The detection of absorbed antigen in the liver is however fraught with many difficulties. Although microscopic techniques e.g. immunofluorescence, autoradiography or visual identificiation (Joel et al 1978) may be used to detect absorbed antigen, these are highly subjective, relatively insensitive, and at best semi-quantitative, and hence are not widely used. On the other hand, there are enormous problems associated with trying to extract the antigen without damaging it or affecting its label; there is also the problem of degradative anzymes during processing or storage of the tissue (Parker 1978). However, Ravin et al (1960) successfully used phenol to extract E. Coli lipopolysaccharide (previously absorbed from the gut) in the livers of rabbits, and Hemmings and Williams (1978) obtained absorbed I^{125} - protein in the supernatant of macerated rat tissues following high speed centrifugation. This latter approach

is unusually simple, and is further discussed in 6.1.

In absorption studies in the intact animal, there are 2 ways to minimize the problem of the sequestration of absorbed antigen by tissues (viz. the liver). Firstly, the antigen can be collected at points before it reaches the liver using thoracic duct or portal vein cannulation. While this has been done in the rat (Warshaw et al 1974), such surgical manipulations are more difficult to perform in smaller animals like the mouse; furthermore, how much the trauma affects the physiological well-being of the animal and hence the intestinal absorption may be a problem. Secondly, the liver function can be blockaded (as with carbon) or destroyed (as in cirrhosis induced with carbon tetrachloride), but this again may affect the intestinal absorption process itself; this technique has not been attempted in experimental absorption studies.

2.2   The Choice of Antigen

The following considerations govern the choice of antigens in oral absorption studies:-

(i)   They should be easily identifiable; by their colour or structure e.g. pollens and spores (Jorden & Linskens 1974), starch grains (Volkheimer 1976), latex particles (LeFevre et al 1978) and carbon (Joel et al 1978); by their activity e.g. insulin (Danforth & Moore, 1959) and horseradish peroxidase (Cornell et al 1972; Owen 1977) or by other properties e.g. ferritin, which is electron-dense (Bockman & Winborn 1966). Many of these are thus identified microscopically. This technique is unfortunately

subjective, semi-quantitative at best, and limited in scope. This property is less important today since immunological tools of identification and radiolabelling are available.

(ii) They should be stable in the gut and body; endotoxin, latex particles, carbon and ferritin for example, are more resistant to degradation than protein antigens.

(iii) They should be available in substantial quantities since the absorption is low.

The albumins, which are available commercially and are easily tagged or identified, are consequently the most popular antigens used in absorption studies. The suitability of their use in absorption studies was recently criticized by Hemmings and Williams (1978). We are however more interested in bacterial antigens as they best relate to the oral immunization studies done in the department here. The two main antigens used are S. Adelaide flagellin (FLA) and V.cholerae Boivin Antigen (BA). Both FLA (Nossal et al 1964) and BA (Horsfall and Rowley 1979) have been shown to be good immunogens (systemically for FLA and orally for BA). The in vivo behaviour of these antigens together with the methods and difficulties of preparing them are discussed in Chapter 5.

2.3 The Use of Cold Antigens

Where possible, cold antigens are used in absorption studies primarily because of the ease in their handling and the complications often associated with radiolabelled antigens. The basic problem here lies in devising assays sensitive enough to detect the minute amounts of absorbed material in the circulation.

While microscopic techniques are too insensitive and subjective, biological assays in general are also insensitive and are restricted to certain antigens only. The limulus lysate test for endotoxin is sensitive (0.25 ng/ml) (Rastogi et al 1977) but its specificity has been questioned (Elin & Wolff 1973). Immunological assays are versatile, sensitive and specific, and are thus widely used. The most common form is direct precipitation with specific antibodies in gels (Andre et al 1974; Worthington et al 1974; Brandtzaeg & Tolo 1977). While this reaction is specific and reliable, its sensitivity is poor (about 1 µg). Higher sensitivities can be achieved with inhibition reactions, as in radioimmunoassays; these sensitivities however, vary considerably among different systems and workers. Andre et al (1974) could detect only 1 µg of HSA in their system involving separation by electrophoresis, while Swarbrick et al (1979) using a double-antibody precipitation approach, could detect as little as 50 pg/ml of ovalbumin in serum. In all studies using these assays, the possibility of new determinants being generated from the in vivo degradation of the native antigen was not considered, since antiserum raised to the parent molecule was used in the assay. It may be more appropriate to use antisera raised against the digested antigen in order to detect new antigenic fragments. In this context, it is interesting to note that the maximal inhibition of the precipitation of sperm whale myoglobulin using antisera raised to it, by six chymotryptic peptides, was no more than 15% (Crompton & Wilkinson 1965).

2.4   The Use of Radiolabelled Antigens

A later aproach in absorption studies is the use of

radiolabelled antigens, which has became popular since the 1960's. An advantage of this method is that less antigen is required to produce measurable absorption since high specific activities can be achieved. Furthermore, the absorbed material can be easily traced by direct counting of the radioactivity. However, it is necessary to separate the free label from protein-bound label. This is easily achieved by dialysis, precipitation, gel filtration or gradient centrifugation. However, precipitation with acids (as used by Hemmings & Williams 1978) irreversibly destroys the antigen and also non-specifically brings down free I^{125} in the presence of tissues (discussed in 6.1). It is also necessary to substantiate that the protein-bound label is the original antigen (or part of it) and not free label bound to serum or tissue proteins of the host. This can be verified by precipitation with specific antibodies directly in solution (Hemmings & Williams 1978), in gels with autoradiography (Warshaw et al 1974), together with a second antibody (Hunter 1973b), with 7% polyethylene glycol (Digeon et al 1977; Creighton et al 1973) or using 50% ammonium sulphate (Minden & Farr 1973). The latter two are the simplest, and they also enable one to detect preformed circulating immune-complexes of the absorbed antigen in the host (Dixon et al 1958). The amount of absorbed label (even after separation from free label) that is precipitable by antibodies is usually small e.g. 6 - 20% as observed by Hemmings & Williams (1978); without knowing the efficiency of the precipitation of the system and the background (control) precipitation, it may be misleading to extrapolate from this the extent of antigenic absorption. Sometimes the absorbed label is merely characterized in gels or gradients (Warshaw et al 1974; Walker et al 1977; Thomas & Parrott 1974) and its identity is thus related to that of the original

macromolecule; the danger here is the possibility of free label (e.g. I^{125}) released _in vivo_ incorporating into host proteins that are of similar molecular size as the original antigen (Tong et al 1954; Rippe _et al_ 1974).

Though antigens can generally be heavily labelled with radioactivity, care must be taken that this does not denature the antigen or affect its physico-chemical properties. For example, radiation damage of proteins is common (Hunter 1973a) and the tyrosine residues to which I^{125} or I^{131} binds may be critical for immunogenicity, as with _S. adelaide_ flagellin (see Parish and Ada 1972). Similarly, the poor uptake of Cr^{51} endotoxin in dogs as observed by Sanford & Noyes (1958) has been attributed to the binding of the label to Lipid A, which presumably is important in the absorption of the endotoxin (Ravin et al 1960).

Consequently, the requisites of a good radiolabel for use in oral absorption studies are:-

(i)   It must be incorporated to give high specific activities (at least microcuries/mg protein) in order to conserve the use of antigen and for the antigen to be detected easily;

(ii) The label must be stable under the conditions in the gut (where gastric acidity and digestive enzymes prevail) and in the body generally;

(iii) The label must be measurable in the presence of body fluids and tissues, and

(iv) There should be no metabolism of the label in the body.

Other considerations include the cost, the ease of

incorporation, the ease of disposal (and its half-life) and particularly in clinical studies, the safety in the usage of the label.

There is no single radiolabel available that meets all these requirements, though the commonly used ones in absorption studies are Cr^{51}, I^{125} and H^3.

Cr^{51} binds non-specifically to endotoxin and is reported to be stable (Rippe et al 1974; Nolan et al 1977). It is easily traced in body fluids and tissues since its gamma-emission is only minimally quenched. It is however difficult to achieve high specific activities with this label. Both Sanford & Noyes (1958) and Braude et al (1955) obtained no more than 0.2 μCi/mg, labelling their endotoxin with $Cr^{51}Cl_3$.

The difficulty in achieving high specific activities is also encountered with Co^{57} (see Kane et al 1974) and S^{35}. The latter has not been used in oral studies, but is very attractive. It is easily coupled to protein antigens by diazotization with S^{35} - sulphanilic acid for instance; the coupling efficiency with BSA or keyhole limpet haemocyanin could be 35-40% (Williams & Chase 1967). Its linkage is stable (Marger and Tarver 1957; Garvey & Campbell 1958), its half-life is ideal (89 days) and it can be conveniently counted in a Geiger-Muller counter or liquid scintillation counter with high efficiency (90%).

The use of tritiated antigens in oral studies has not been popular, mainly because of the difficulties in tracing them in tissues, due to quenching. Moreover, the counting efficiency of

tritium in liquid scintillation is usually less than 60%. However, the uptake of H^3 - BSA in the lymph and plasma of rats was successfully measured by Warshaw et al (1974). Labelling of the antigen is usually done by exchange with tritiated water (Evans et al 1963) or with tritium gas (Wilzbach 1957); the set-back here is the lability of the H^3 atom (particularly in certain positions of the antigen), which may be displaced in vivo.

By far, the radiolabel most commonly used in absorption studies is I^{125} or I^{131}. Labelling is easily achieved externally using either the chloramine-T method (McConahey & Dixon 1966) or the peroxidase method (Matzku & Zoller 1977), and activities of 1 $\mu Ci/\mu g$ are commonly obtainable. The times of decay are convenient (the half-lives of I^{125} and I^{131} are 60 days and 8 days respectively), and they can be counted directly in tissues in a gamma-counter or whole body counter without significant quenching. There is thus great ease in the handling of this label. The serious shortcoming however, is the lability of the C-I linkage. Deiodination is not only encountered in vivo (Marger and Tarver 1957) due to enzymes in the gut (Parkins et al 1960; Foster & Gutman 1930)[*], liver (Tong et al 1954), and other tissues (Stanbury 1957), but also on sheer storage in aqueous solution at 4^oC (personal observation; LeFevre et al 1977). For this reason, the suitability of iodinated proteins in oral absorption studies in vivo must be questioned. Deiodination is a serious problem, as large amounts of free iodine can be recovered from the circulation (Parkins et al 1960; LeFevre et al 1977) which certainly complicates detection of the labelled antigen. The possibility of re-utilization of the liberated iodine by the host further questions the validity of their use (Tong et al 1954). The loss

[*](Cox & Hinchliffe 1961)

of label from the absorbed protein could result in an underestimation of its rate of absorption (as observed by Jones 1977), and this may also explain the observations that little (Thomas & Parrott 1974) or no (Parkins et al 1960; LeFevre et al 1977) antigen-bound iodine was found in the circulation, despite the presence of large quantities of free iodine. On the other hand, Hemmings & Williams (1978) recently claimed that as much as 40% of their I^{125} proteins was absorbed as high molecular weight material; this interpretation requires caution and is discussed in 6.1.

Finally, the possibility of using C^{14} and P^{32} as markers merits some consideration, though this has not been exploited. Both can be incorporated internally, e.g. in growing bacterial cultures. The difficulties of detecting H^3 are also met with C^{14}, though the stronger emission of the latter allows a counting efficiency of 90% in liquid scintillation. While H^3 has a half-life of 12 years, that of C^{14} is 5,400 years, and this makes both unsuitable for clinical use. P^{32} on the other hand, has a useful half-life (14.5 days), and it can be easily counted in a Geiger-Muller counter. However, the presence of phosphatases in vivo can also lead to problems.

2.5   The Choice of in vivo Models

In experimental studies on the intestinal absorption of macromolecules in vivo, the rat is perhaps the most commonly used animal (Warshaw et al 1974; Hemmings and Williams 1978; Worthington et al 1974). It is just large enough for intricate surgery to be performed e.g. thoracic duct cannulation and

portacaval shunt, and the absorbed antigen may be recovered in a suitable volume of serum or lymph. Dogs (Sanford and Noyes 1958) and rabbits (Kingham et al 1976) are sometimes used, while limited studies have been done in mice (Swarbrick et al 1979). The small size of the latter certainly poses problems in terms of surgical manipulations and in the recovery of the absorbed material in body fluids. Nevertheless we ourselves have adopted the mouse because (1) our oral immunization studies, including those on cholera and typhoid, are done in it, (2) it is easy to handle, and (3) it is available in large numbers and varieties of strain.

2.6   In vitro Models

Due to the formidable difficulties inherent in absorption studies in the intact animal (in view of the extensive degradation of the antigen in the gut, and the poor recovery of the absorbed material), in vitro models are popular substitutes (Walker et al 1972a; Nolan et al 1977; Brandtzaeg and Tolo 1977). The amount of macromolecular material absorbed in these models can be increased to suitable levels by increasing the surface area of the mucosa (e.g. bigger or more sacs), the antigen concentration, or the time of incubation; furthermore, the absorbed material can be recovered in total. Another advantage of these models is the possiblity of modifying the microenvironmental conditions of the gut (e.g. removal of the mucus or enzymes, and the passive adsorption with specific antibodies) and to study these changes on the absorption. The main criticism of these in vitro models however, is how well they relate to the intact animal, since any handling of the intestine, no matter how gentle, might affect its physical and functional integrity, including that of the mucous

component. Moreover, the in vitro conditions used to maintain the viability of the gut (95% oxygen) does not simulate the microaerophilic or anaerobic conditions of the intact gut. The answers provided by these models are also limited; for instance, the final fate and importance of the absorbed material requires definition in the intact animal.

2.7   Summary

The question of whether amino-acids were absorbed from the intestine was only resolved by more sensitive techniques and the problem of detecting them in the circulation was illustrated by studies showing their rapid disappearance here. The absorption of macromolecules must be many fold less than that of amino-acids from the same protein, and consequently the difficulty in demonstrating it must be greater. Highly sensitive methods are thus required to detect the absorbed antigen. However, these assays are sometimes difficult to establish with some systems, while on the other hand, they may not be suitable for use with biological samples. As with amino-acids, the problem that the bulk of the absorbed antigen ends up in the tissues is real and not easily solved; there is the difficulty of detecting it here or in extracting it. Liver by-passes in the intact animal or in vitro models are possible solutions, but these are often criticized on physiological grounds. The use of radiolabelled antigens may enable their detection in tissues, but there exists the difficulty in showing that the label remains associated with the original antigen. There is no single label available that is ideal for use in oral absorption studies.

The difficulties of these studies have in some ways limited the scope and goals (including the methodologies used) in the field. Many studies have used similar antigens, models and approaches and arrived at similar conclusions, without really shedding new light to the problem.

3.   CHAPTER 3. PERSONAL APPROACHES TO THE PROBLEMS IN ORAL

ABSORPTION STUDIES

The problems encountered in the early studies on the intestinal absorption of BA and FLA in vivo are related in this introductory chapter, and the solutions to some of these are outlined.

3.1   Attempts at Developing Sensitive Immunoassays for Detecting

Antigen

The immunoassays examined for detecting FLA and BA were basically inhibition reactions derived from passive haemagglutination and radioassays. The latter were varied according to whether the antigen, the primary antibody or the developing second antibody was labelled with I^{125}, and to the mode of separation of the free labelled antibody (or antigen) from the complexed label (see Hunter 1973b).

3.1.1   Inhibition of passive haemagglutination

Glutaraldehyde-fixed, tanned sheep red cells (SRBC) sensitized with antigen (FLA or BA) were used at a concentration of 4.8 x 10^5 cells/microtitre well, containing 4.6 - 9.9 ng antigen (see MATERIALS and METHODS 4.6.2). With FLA, this system could detect 1.2ng mouse specific antibody (Figs. 3.3 and 4.1); using 2.2ng of this antibody in an inhibition assay, 4.5 - 9.0ng FLA (i.e. 45 - 90ng/ml) could be detected . With BA, the limit of detection was

390ng/ml for a purified preparation (BA#abc) and 460ng/ml for a crude preparation (BA#DBA); the presence of contaminating "inert" material in these preparations resulting in erroneous estimation of the dry weight probably accounts for the poor sensitivity of the system. However, detection of the antigen in biological fluids suffers from the fact that mouse serum inhibits the system, and has to be used at a final dilution of greater than 1:8 in order to give unequivocal results, while thoracic lymph appears to potentiate the agglutination.

In an attempt to increase the sensitivity of this assay, and at the same time lower the subjectivity, the reacted cells were counted in a Coulter counter (Model F) (Bowdler and Swisher 1964; Gibbs et al 1975). However, this only increased the sensitivity of the test slightly (Table 3.1). In another approach, the reacted cells were treated with I^{125} goat antibody directed against the primary antibody (mouse light chain) to determine the amount of antibody bound (see 4.6.5). However, at the low concentration of cells used for haemagglutination, the superimposed radioassay gave poor resolution.

3.1.2   Inhibition of the Farr assay

The inhibition of the precipitation of I^{125}-BSA using minimal amounts of rabbit anti-BSA together with 45% saturated ammonium sulphate (4.6.4) was studied. The sensitivity was poor (1.5 μg antigen); this may be expected since the amount of antiserum used to produce reasonable resolution was large. This had discouraged further studies with other antigens.

Table 3.1   Electronic quantitation of the inhibition of

haemagglutination using the COULTER COUNTER (Model F).

SETTINGS:
Aperture   = 0.5
Attenuation = 8

Exp	Inhibitor (FLA) ng/ml	Visual pattern	$COUNTS \times 10^{-2}$ at different thresholds (T)		
			T=17.5	T=20	T=25
1	360	−	193	188	176
	180	−	131	132	119
	90	−	101	97	94
	45	−	(14)	88	81
	27.5	+	83	85	85
	13.75	+	77	76	77
	6.88	+	79	76	75
	3.44	+	77	77	78
			T=18	T=35	T=45
2	360	−	128	62	46
	180	−	98	41	25
	90	−	86	35	22
	45	−	70	27	18
	27.5	+	68	28	18
	13.75	+	63	28	18
	6.88	+	61	29	19

+ haemagglutinated   − inhibited reaction

3.1.3   Inhibition of binding of labelled antigen to specific

antibody : separation on paper

The extremely sensitive method of Yalow and Berson (1964), with which they detected less than 1ng/ml insulin in plasma, was studied in an attempt to detect BA. In principle, this is simple and attractive; when the labelled antigen is applied to an absorbent paper and then made to migrate (e.g. by electrophoresis), it will stay absorbed at the origin unless it is complexed with antibodies, when it will move with them. This technique thus requires special properties of the paper and antigen, and the latter must be different from immunoglobulins in electrostatic properties at least. In a preliminary attempt, the individual migration of I^{125} BA and I^{125} goat antibody on Whatman papers 3MM and 41 under the influence of normal mouse serum was studied. As shown in Fig. 3.1, the separation of these markers was poor. Hence, greater refinement of the system, probably requiring a more suitable paper, is needed before it can be used to detect BA.

3.1.4   Inhibition of the binding of I^{125}-specific antibody

to antigen-sensitized RBC

The separation of the label (antigen or antibody) that is complexed, from that which is free, may be better achieved by centrifugation using an insolubilized antigen or antibody, than that obtained by precipitation or on paper. It is easier to insolubilize the antigen (without loss of antigenicity) than to

Fig. 3.1

Preliminary attempt to separate antigen from antibody on (A) Whatman 41,
(B) Whatman 3MM papers.

The I^{125}-antigen and the I^{125}-antibody were mixed separately with
different dilutions of normal mouse serum. These were separately
spotted on 3 x 10 cm strips of Whatman papers 41 and 3MM. When dried,
the end of the paper near the origin (spotted with the labelled compounds)
was dipped into 0.1M Veronal buffer pH 8.2, 25°C. The flow of buffer
across the paper was continued till the waterfront almost reached the
other end of the paper (about 15 min). The paper was then dried and the
origin and the waterfront were cut and counted. The amount of radio-
activity that migrated from the origin was thus determined.

■   antigen (BA)

O   antibody (goat anti-mouse Ig)

Fig 3·1
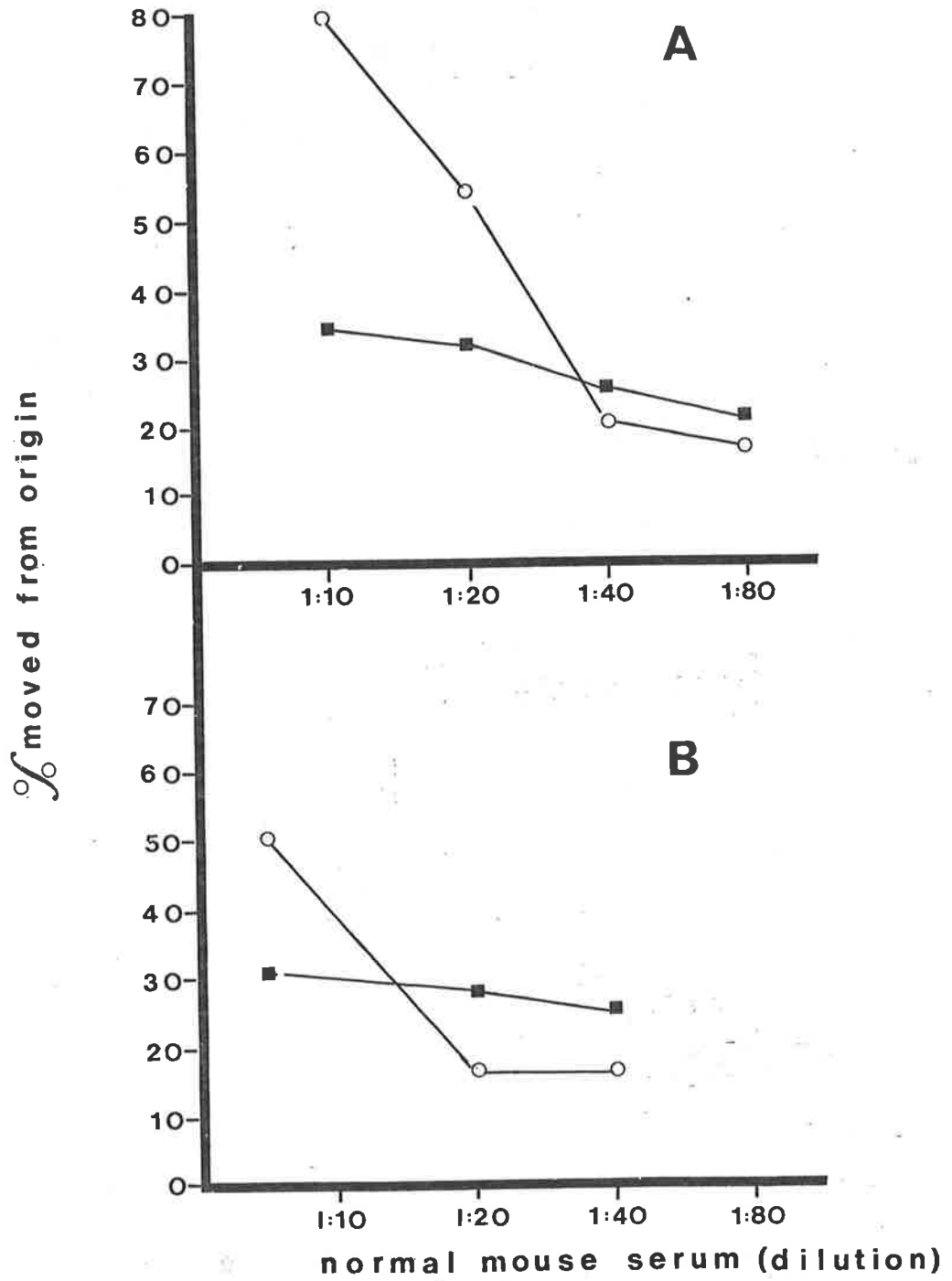
insolubilize the antibody (without loss of avidity and function). Though we could not insolubilize BA by cross-linking with 1% glutaraldehyde (Avrameas and Ternynck 1969), absorbing it to glutaraldehyde-fixed red cells (4.6.2) was satisfactory. This was preferred over fixed whole bacterial cells, as the amount of antigen used in the red cells is limited and could be quantitated.

Purified mouse anti-BA antibody (4.5.1) labelled with I^{125} (4 μCi/μg), was reacted at different concentrations with varying numbers of antigen-sensitized cells (BA-RBC). The amount of I^{125} antibody bound was determined (Fig. 3.2). The binding was poor, and this was probably due to inactivation of the antibody during purification and iodination. In the inhibition studies used for quantitating antigen, BA-RBC containing 640ng antigen was used in order to achieve good resolution. The amounts of I^{125} antibody used were 0.38ng and 3.8ng. However, at both concentrations, the amount of antigen detectable by this assay was about 30x poorer than that achieved by the inhibition of haemagglutination.

3.1.5 Inhibition of binding of primary (mouse) antibody to antigen-sensitized cells or specific bacteria, and developed with a secondary labelled-antibody (I^{125} goat anti-mouse IgLC)

The purification and iodination of the primary antibody in 3.1.4 could account for its poor binding and the poor sensitivity of the system. In the present attempt, the antigen-specific antibody was not labelled, but its presence on the immunoabsorbent was detected

Fig. 3.2

Binding of I^{125}-mouse anti-BA antibody by different numbers of BA-sensitized red cells.
Expressed as (A) total counts bound, and (B) % counts bound

SRBC coated with $ca.$ 640 ng BA (●) were serially diluted in 7 two-fold steps (using PBS containing 1% normal mouse serum), so that the highest dilution (△) contained about 10 ng BA.
The dilutions of SRBC-BA were shaken at 37°C for 90 min with each of 4 concentrations of I^{125}-anti-BA (0.28 ng - 3.8 ng).
The cells were washed twice in the diluting buffer before counting.

Fig. 3.2
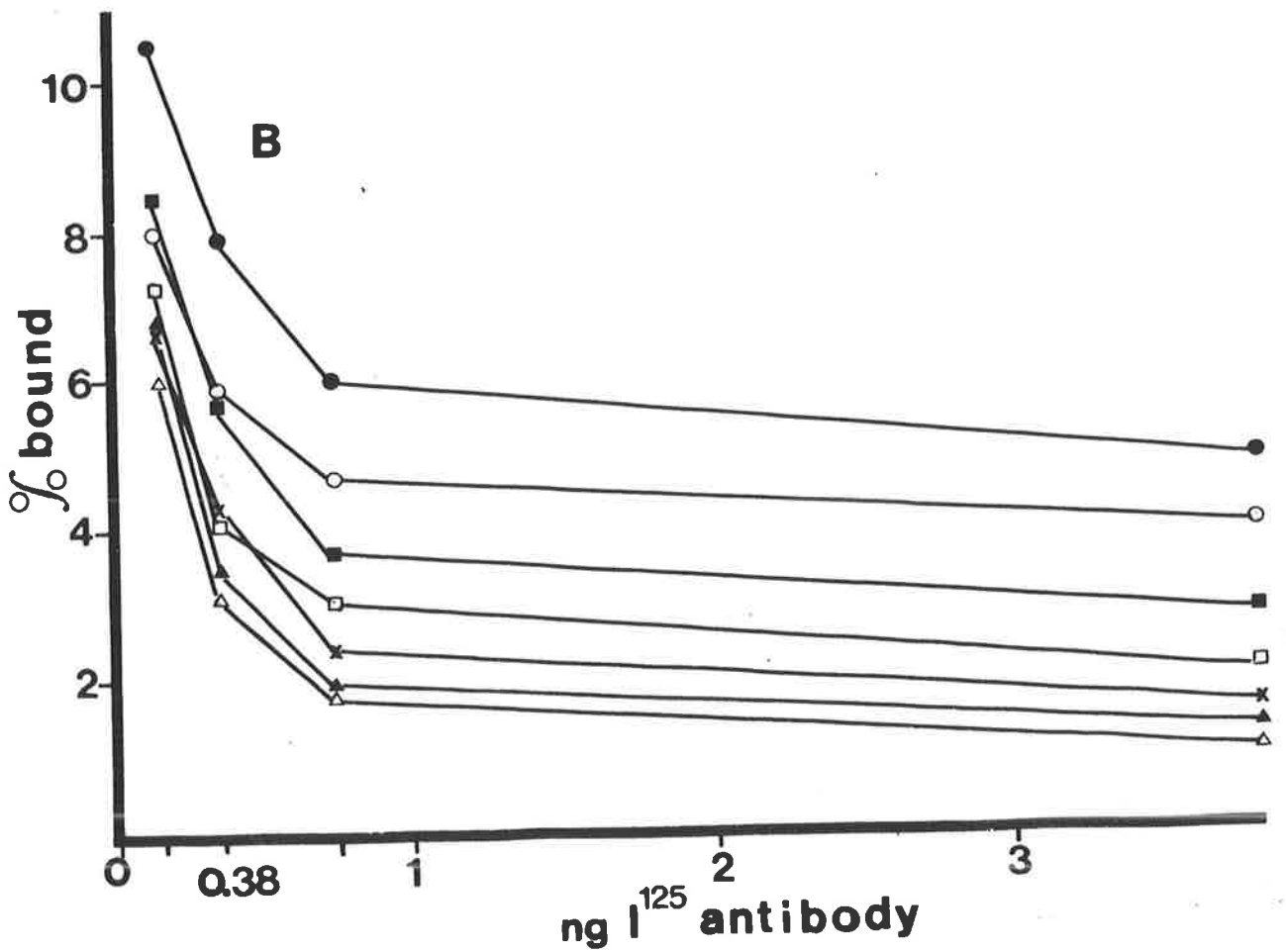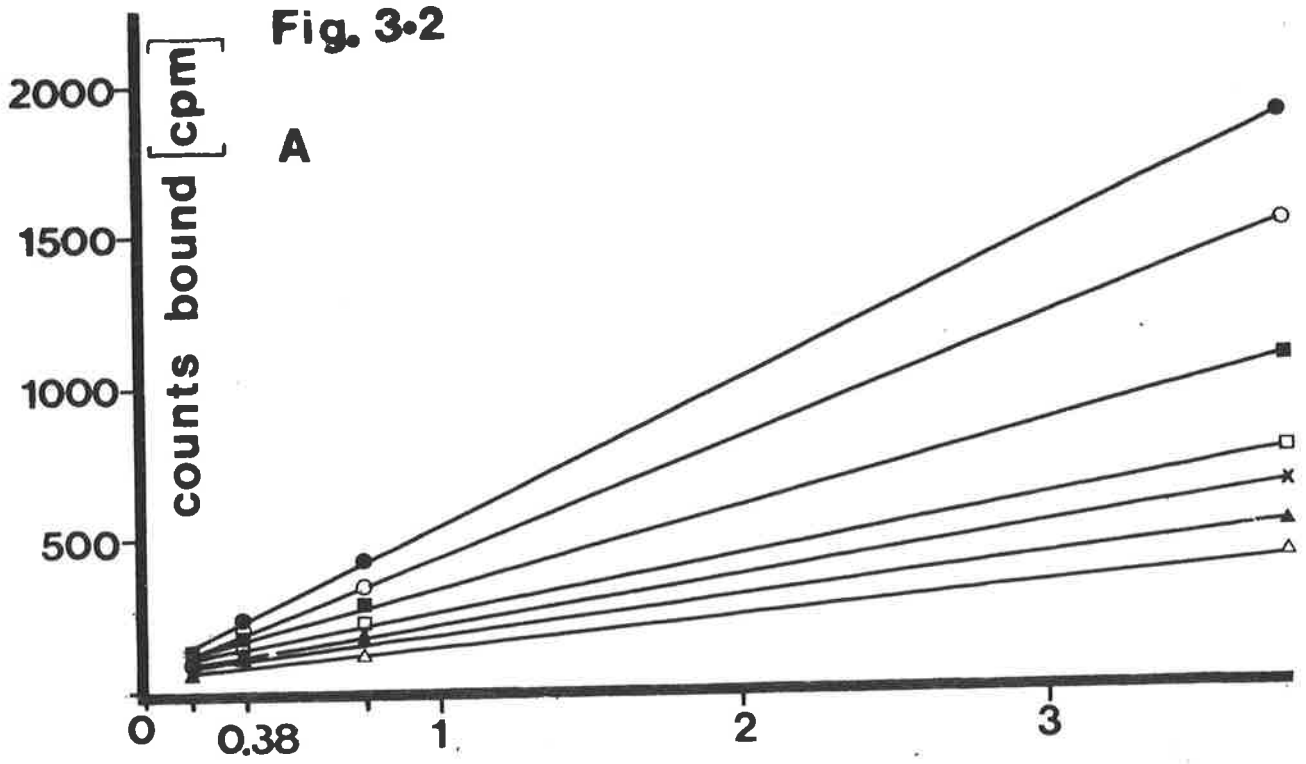
using a labelled antibody directed against it, as described in 4.6.5. Thus, using FLA-RBC or fixed S.adelaide bacteria as the insoluble absorbent, purified mouse anti-flagellin as the primary antibody, and I^{125} goat anti-mouse IgLC as the detector, the binding curves obtained are shown in Fig. 3.3. It is evident that while 2.25ng of primary mouse anti-flagellin antibody could be used in the inhibition of haemagglutination, about 9ng of the antibody was required in radioimmunoassays using either 10^8 S.adelaide cells or 1% FLA-RBC (about 100ng antigen) in order to produce reasonable resolution. Under these conditions, the lowest amount of cold inhibitor (FLA) detected was 725ng/ml. Thus, this assay is about 8x less sensitive than the inhibition of haemagglutination for detecting FLA.

3.1.6 Discussion on the choice of immunoassay to detect cold

antigen

Of the immunoassays examined to detect BA or FLA, the inhibition of passive haemagglutination was the most sensitive. It could detect 45-90 ng/ml FLA and 390-400 ng/ml BA in buffer, although in serum it was necessary to use a dilution greater than 1:8 to avoid interference of the system. This assay is simple and reproducible. It is also more sensitive than precipitin tests commonly used in absorption studies, and its use in this area will be discussed in 6.3.2.

The main reason for the greater insensitivity of radioimmunoassays compared with assays employing the inhibition of haemagglutination in the systems studied is perhaps because the

Fig. 3.3

Binding curves of FLA anti-FLA systems using the double antibody
radioimmunoassay.

Different amounts of purified mouse anti-FLA antibody were reacted
either with 5 x 10^8 glutaraldehyde-fixed *S. adelaide* (□) or
FLA-sensitized SRBC, containing about 100 ng FLA (○). After
incubation for 18 h /4°C, the cells were washed twice and then
reacted with I^{125}-goat anti-mouse IgLC (10,000 cpm/17 ng) for 30 min /25°C
in a shaking water bath. The cells were washed twice again and counted.

(The haemagglutination titre of the anti-FLA preparation was
determined previously using FLA-SRBC, containing 4.6 ng antigen).

Fig. 3·3
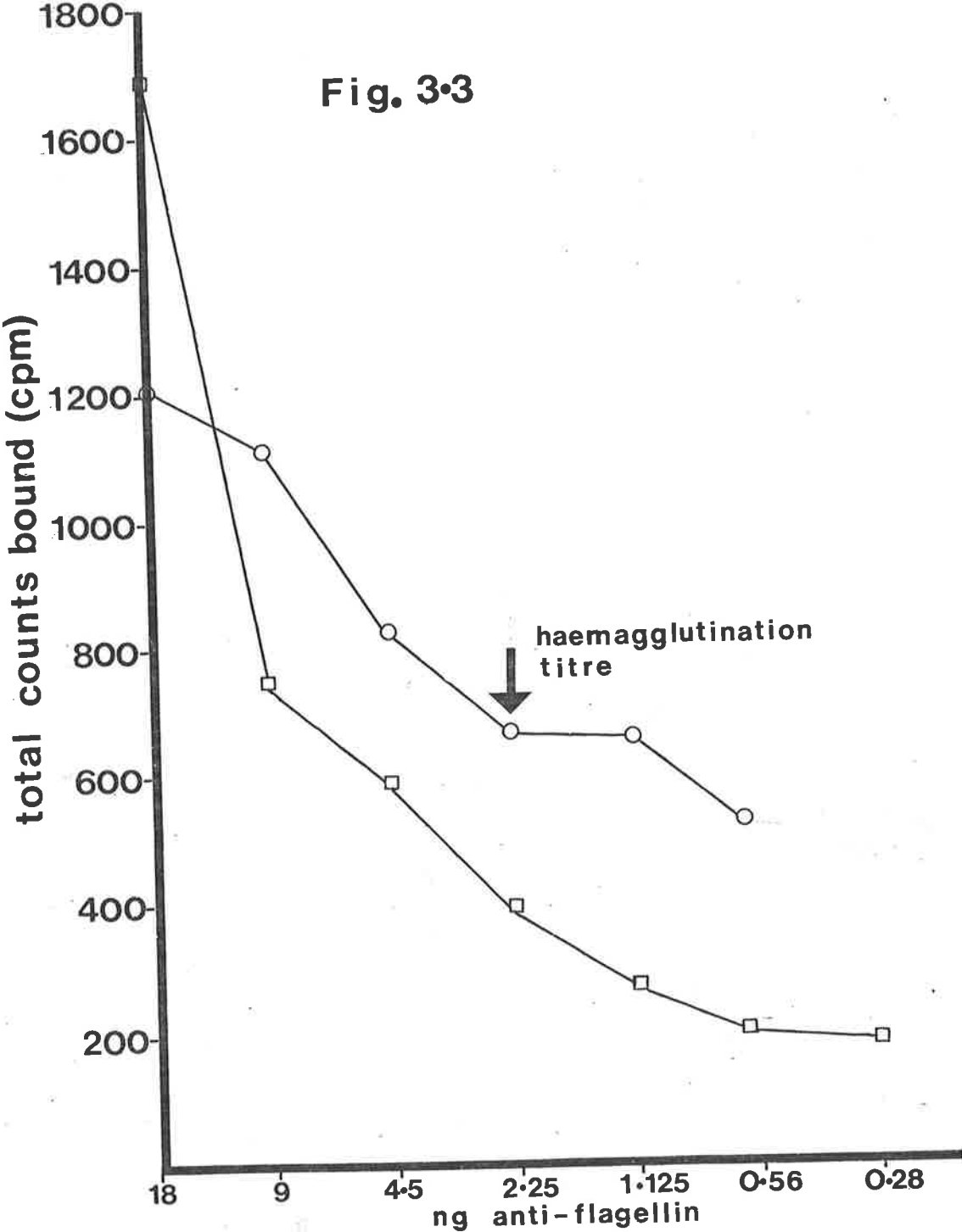
latter required less antibody and antigen to produce a positive reaction. This may be due to the poor affinity of the antibody (Tosi and Celada 1974; Parker 1976). Moreover, mouse antibody may not be ideal, as it is not only difficult to obtain, but it also appears to be extremely fragile in our fractionation procedures (compared with goat antibody for instance). Highly sensitive radioimmunoassays have nevertheless been reported (Yalow and Berson 1964; Swarbrick et al 1979; Hunter 1973b).

3.2 The Use of Radiolabelled Antigens

There are both advantages and disadvantages in using either cold antigen or radiolabelled antigen in oral absorption studies. In the latter case, the antigen can be traced in tissues (this is difficult with cold antigen), and there is no need for a sensitive immunoassay so long as the specific activity of the antigen achieved is high. The main problem here is the release of free label from the antigen or the metabolism of the label in vivo, which could complicate the detection of the labelled antigen. Our studies with radiolabelled antigens are introduced in this section.

3.2.1 The use of I^{125} labelled antigens

Radioiodinated proteins have been widely used in oral absorption studies with variable success. Consequently, our first attempt was made using I^{125} BSA, I^{125} BA and I^{125} FLA. These studies were disappointing, since more than 90% of the radioactivity absorbed in both circulation and tissues was dialysable. Further, the non-dialysable label could not be

precipitated by specific antibodies to any of these antigens in the Farr assay. The shortcomings with iodination are further discussed in 6.1. We have also attempted to obtain a stable iodinated preparation thus. I^{125} BA was incubated with normal mouse intestinal juice in a dialysis bag at $37^{o}C$ and kept stirred in a beaker of buffer (PBS, pH7.5). Free I^{125} was progressively released to the outside; when, eventually after 18 hours, the residual non-dialysable compound was fed to mice, it was deiodinated as readily as the untreated antigen.

3.2.2   The use of Cr^{51} labelled antigens

The reported in vivo stability of Cr^{51} compounds (Nolan et al 1977) encouraged us to investigate their use. However, the labelling of S.adelaide lipopolysaccharide with $Na_2Cr^{51}O_4$ in 0.05 Mphosphate buffer (pH 7.5) at $37^{o}C/2$ hours, was extremely poor (0.002µCi/mg LPS). This, together with the low incorporation also obtained by others e.g. Sanford and Noyes (1958), had discouraged further use of this label.

3.2.3   The use of H^{3}DNP-labelled antigens

In a novel approach, we have successfully used BA and FLA labelled with H^{3}dinitrophenyl (DNP) in absorption studies in the intact animal; this is elaborated in 6.2. This idea was borne from a number of considerations. Firstly, the conjugation is simple and strong (Sanger 1946). Secondly, 1-Fluoro-2,4-dinitro {3,5-3H}benzene (H^{3} DNFB) is available commercially with high activities (10-30 curies/mmol) (Radiochemical Centre, Amersham). Over 90% of the tritium activity is distributed over the 3' and 5'

carbon positions. At these positions, there is minimal electrophilic exchange (Dr. G. Gream, personal communication; see Sykes 1975). Consequently, the possibility of isotopic exchange here is much less than with compounds labelled by conventional means (Evans et al 1963). Thirdly, the availability of an IgA myeloma with anti-DNP activity (MOPC 315) makes this label a suitable marker for studying the effect of specific antibody on the absorption of antigen.

Though BA and FLA are easily dinitrophenylated with cold DNFB, the incorporation that was initially achieved with H^3 DNFB was poor. However, the addition of cold DNFB to increase the final concentration of the reagent to 2.5mg/ml enabled a coupling efficiency of about 30%. The antigens were not denatured (e.g. due to over -dinitrophenylation) under these conditions, as the dinitrophenylated antigens were precipitated just as well as the parent compound by specific antibodies. Another problem with the use of H^3DNP as a marker (or with tritium in general) is the difficulty in tracing it in tissues. However, this may be facilitated by the use of a sample oxidiser (Packard) which efficiently (claimed to be 99.1%) burns up the tissue and converts associated tritium to tritiated water, which is automatically collected in the scintillation fluid. (In principle, this is similar to the Schoniger technique (1955) used by Evans et al 1963). However, unlike the direct counting of iodinated compounds, this process is labour and time consuming; moreover, the equipment is not readily available.

3.3   The Use of I^{125}-Labelled Proteins Administered

Intravenously

Though radioiodinated proteins are readily deiodinated when given orally to mice, they are relatively stable when given intravenously, as recovered in both tissues and circulation, for as long as 2 hours post injection (see 5.3, 5.4). This fact, together with the great ease in the handling of iodinated compounds, is exploited in the two approaches mentioned below.

3.3.1 The use of I^{125} anti-BA antibody given intravenously

to detect the oral absorption of BA in vivo

The rationale in this particular approach, to detect any cold BA absorbed using I^{125}-labelled specific antibody given intravenously, is that in the presence of the absorbed antigen, there will be less of the labelled antibody in the circulation and more in the liver, due to immune-complex formation. This technique is theoretically attractive since it directly measures the total amount of antigen absorbed (even in tissues), unlike other approaches which merely examine a sample of the serum. In practice however, there were serious difficulties in perfecting the assay. There was a high uptake of the I^{125} homologous antibody in the liver when given alone to normal mice. The uptake was about 8% of the dose given, in livers perfused with excess buffer (PBS) containing 1mM KI. The I^{125} antibody was further purified on Sephadex G75 to remove any fragments, and then centrifuged at 32,000g/1 h/4°C. While the centrifugation did not decrease the background uptake of the labelled antibody by the liver, an in vivo purification of the antibody (by recovering it

from the plasma of normal mice 20 minutes after injecting it intravenously) reduced this uptake by 50%. However, this $\underline{in}$ $\underline{vivo}$ purification resulted in great loss (90%) of the label due to poor recovery, and the preparation not purified by this means was thus used in the absorption experiments discussed in 6.3.1. It was necessary to dilute the I^{125} antibody with cold antiserum (MAB#1276), so that the amount of specific antibody injected was about $1\mu g/0.2ml$ (final specific activity, about 50cpm/ng). This increased the sensitivity of the assay for detecting small amounts of the antigen ($10\mu g$ - $200\mu g$) given intravenously with the I^{125} antibody. In retrospect, the sensitivity of the assay could perhaps be increased if (1) the soluble complexes formed in the plasma were removed e.g. with polyethylene glycol before determining its free I^{125} antibody content; and (2) a more avid antibody was used in higher specific activities in the detection. The index (LP = liver cpm/plasma cpm) was used to gauge the presence of antigen in the system rather than merely determining the radioactivity in the plasma or liver, as this is not only more sensitive but also takes care of any error in the dose of I^{125} antibody administered.

3.3.2   The use of I^{125} BSA given intravenously to monitor gut

permeability

It may not be valid to use I^{125}-proteins administered orally to study their uptake into the circulation because of the problem of deiodination already mentioned. However, the reverse transport i.e. from the circulation to the gut, of I^{125} proteins may be studied without similar criticisms, if it does not matter what the

nature of the transported protein in the gut is. Thus, we have used the leakage of I^{125} BSA from the circulation to the gut of mice given various treatments to gauge the intestinal permeability to unrelated antigens (7.3). The validity of this approach is supported by a similar *in vitro* technique, where the transport of I^{125} BSA was monitored from the mucosal surface to the serosal side (7.3.3.). *(See also 7.6).*

3.4   The Immunogenic Detection of Absorbed BA

Numerous biological assays have been used to detect absorbed macromolecules. For instance, the toxicity of endotoxin, especially when given with Actinomycin D (Shute 1977) has been exploited. This test is however, not specific. We have taken advantage of the immunogenicity of BA and tested its presence by its ability to prime normal mice (orally and intravenously) to a standard i.v. booster dose of the antigen (100ng) 3 weeks later. This test could detect as little as 1ng of antigen, and it is discussed more fully in Chapter 8.

3.5   Summary

In this chapter, the difficulties that we enountered in attempting to measure the intestinal absorption of macromolecules in the intact animal, together with our approaches to these problems are discussed. The inhibition of haemagglutination was the most sensitive assay found for detecting FLA and BA. An *in vivo* technique using I^{125} specific antibody given intravenously to detect orally absorbed BA was also discussed. While radioiodinated antigens were found unsuitable for oral absorption

studies, antigens labelled with H^3DNP were useful alternatives.
A sensitive, immunogenic test for BA was also found. This
utilises the ability of minute amounts of the antigen to prime
mice for an antibody response when boosted subsequently.

4.   CHAPTER 4. MATERIALS AND METHODS

4.1   Animals

4.1.1   Mice

Unless otherwise stated, specific pathogen-free (SPF) LAC mice obtained from the Central Animal House of this University, were used in all studies. In others, germ-free (GF) BALB/C mice bred in the department (Horsfall 1977), were used.

In all oral absorption experiments, including those where the intestinal contents were obtained, mice were starved, but allowed water, overnight. Mice that were immunized orally with specific bacteria and used in some experiments, were obtained by the method described in 4.5.7.

4.1.2   Rats

100 day-old Lewis rats, weighing about 300 gm, were used in some absorption studies; they were not starved prior to the experiment.

4.2   Bacteria

Vibrio cholerae 569B (Clasical, Inaba), originally obtained from the Cholera Research Lab., Dacca, and Salmonella adelaide were used. They were maintained on nutrient agar slopes at $4^{o}C$; the

former was subcultured weekly and the latter monthly.

4.3   Antigens

4.3.1   Boivin Antigen (BA) from V. cholerae 569B

This is described in Chapter 5.

4.3.2   Flagellin (FLA) from S. Adelaide

This is described in Chapter 5.

4.3.3   Bovine Serum Albumin (BSA)

Freeze-dried Cohn's Faction V (Commonwealth Serum Lab.) was used without further purification.

4.3.4   Mouse IgG1 (MOPC 21)

This myeloma protein purified on Sephadex G200, was   a   gift   of Dr. P. Ey.

4.3.5   Lipopolysaccharide (LPS) from S. adelaide

The   method   used   for   preparing   LPS   from   S.adelaide   (grown overnight on solid nutrient agar at $37^{o}C$) by phenol-extraction was as described by Westphal et al (1952).

4.4 Labelling Antigens

4.4.1 Iodination

The method of Greenwood et al (1963) was used. 30 µl of protein (0.5-2.5 mg/ml; previously dialysed against 0.1M phosphate buffer pH 7.5) was placed in a small Durham tube, kept in ice. 2-10 µl NaI^{125} (Amersham, London; 1 mCi/10 µl) was then added with a microsyringe. The reaction was started by adding 15 µl chloramine-T (0.5 mg/ml) and the reagents were mixed constantly with a Finn pipette. After 5 minutes, the reaction was stopped with 30 µl sodium metabisulphite (0.5 mg/ml). The reaction mixture was run on Sephadex G25 to separate the free from bound iodine, or, when many antigens were iodinated at the same time, dialysed first in 100 ml phosphate-buffered saline (PBS) and later in 3 changes of 500 ml PBS.

The specific activities obtained were variable for different proteins and for different preparations of the same protein (Table 4.1). However, the specific activity of each radiolabelled preparation used is mentioned in the text where appropriate.

4.4.2 Tracing iodinated antigens

I^{125} in buffer, whole organs, gut wash-outs and biological fluids was detected directly in sealed tubes in a well-type gammacounter (Packard). Whole mouse carcass and portions of rat carcass were counted in a whole-body counter. No correction was made for any differences between these two counters, or for any

Table 4.1 Results of radioiodination using the Chloramine-T method

PROTEIN	SPECIFIC ACTIVITY $\times 10^{-2}$ cpm/ng	
	average	range
goat anti-mouse IgLC	15	13-26
goat anti-mouse IgA	20	20-30
mouse anti-BA	20	10-30
BA	5	1-10
BSA	7	5-8
FLA	8	8-10

quenching in tissues. Except for Table 6.1, estimations of the radioactivity administered to animals were usually based on a 10 μl sample determined in the well-type counter.

4.4.3   Dinitrophenylation

The dinitrophenylation of BSA, BA and FLA with 1-Fluoro-2,4-dinitrobenzene (DNFB) (British Drug Houses) was essentially as described by Klaus and Cross (1974). 5 ml of protein solution (20 mg/ml; dialysed previously against 2% K_2CO_3) was placed in a 10 ml beaker. It was protected from light with aluminium foil and kept stirred in the cold room (4°C). For high incorporation, 20 μl DNFB in 0.2 ml acetone was added slowly with a Finn pipette, while for lower incorporation, 2 μl DNFB in 0.2 ml acetone was similarly added. After 4 hours, the reaction mixture was dialysed extensively to remove unreacted DNFB.

With H^3 DNFB (Radiochemical Centre, Amersham, London), the approach was modified. This reagent (14.5 Ci/mmol) is benzene-based and has a low concentration (13 μg/ml). It was necessary to increase this concentration with cold DNFB (diluted in benzene) for effective incorporation. Thus, 25 μl H^3DNFB and 5 μl cold DNFB (15 mg/ml) were mixed for use with each lot of antigen. 0.2 ml protein solution (4 mg/ml; previously dialysed against 0.2 M $K_2 CO_3$) was placed in a clean glass tube (1 x 6 cm). 30 μl DNFB mixture was added slowly with a Finn pipette while the tube was shaken vigorously in a vortex. The tube was later sealed, covered in aluminium foil, and shaken in an orbital water-bath (2000 rpm/4°C/4 h). As before, the mixture was dialysed.

The specific activities obtained with BA (#abc) and FLA were about 1-2 cpm/ng. In both cases, the same preparation of radiolabelled antigen was used for all studies.

4.4.4 Tracing H^3 dinitrophenylated antigens

Solutions and plasma containing H^3 DNP-compounds were spotted on glass fibre discs. 10 ml scintillation fluid (Monophase-40, Packard) was added to the dried discs in counting vials; these were counted in a beta-counter (Packard, 60% efficiency).

Whole organs and other tissues containing H^3DNP label were first processed (see 4.7.9), lyophilized, weighed and then burnt in a sample oxidizer (Packard). The efficiency of the oxidiser was determined (using known amounts of H^3DNFB impregnated in cotton wool) to be 18-30%, and corrections for this were made accordingly in the results. The scintillation fluid so obtained (containing associated tritium from the tissue) was clarified if necessary, by low speed centrifugation (1,000g/10 min) before being counted.

4.5 Preparation of Antibodies

4.5.1 Mouse anti-V.cholerae serum (MAB # DIgM, #1276, #LB)

Mice were hyperimmunized with 10^8 live, log-phase V.cholerae 569B; these were given i.p. in saline bi-weekly for 3 weeks. The mice were bled 7 days after the last shot from the retro-orbital

plexus.

Purified mouse anti-BA antibody was prepared from antiserum MAB #1276 using affinity chromatography. A 1 x 15 cm column was made from 2.5 gm CNBr-activated Sepharose 4B (Pharmacia) reacted with 10 mg BA; about 30% of the antigen was coupled (see 4.5.4 for details). The bound antibody was eluted with 3M NaSCN (Edgington 1971) at 4°C and dialysed immediately. (It was denatured more readily with glycine-HCl buffer pH 2.9.) The purified antibody had 10 haemagglutinating units/µg protein (OD_{280}).

4.5.2   Mouse anti-FLA serum (MAF)

This was obtained from mice given 3 x 1 µg flagellin i.p. in saline, 3 weeks apart. Part of the serum was fractionated with 50% ammonium sulphate and purified on a 10 ml flagellin-sepharose 4B column (prepared as in 4.5.4; 40% coupling efficiency). The purified antibody, eluted with 3M NaSCN, had 30 haemagglutinating units/µg protein.

4.5.3   Mouse IgA anti-DNP (MOPC 315)

This myeloma protein was partially purified on Sephadex G200 (kindly provided by Mr. G. Russell-Jones).

4.5.4   Purified goat anti-mouse IgLC (light chain) and anti-IgA

Goat anti-mouse IgM serum was obtained by hyperimmunization with 6 x 1 mg weekly doses intramuscularly (kindly provided by Dr. D. Horsfall). Purified goat anti-mouse IgLC (light chain) antibody

was obtained by fractionating this serum on an affinity column containing mouse IgG. (The IgG was prepared from normal mouse serum by caprylic acid precipitation - Steinbuch and Andran, 1969). The column was prepared as follows: 2 gm Sepharose 4B - CNBr (Pharmacia) was activated with 3x100 ml 1 mM HCl, and then resuspended in 15 ml 0.1M borate buffer pH 8.0. 24 mg mouse IgG (5 mg/ml; previously dialysed in the same buffer) was added to the gel. This was stirred gently at room temperature ($25^{o}C$) for 3.5 h; the gel was then filtered and washed with 250 ml 0.1M borate buffer (pH 8.0). (Spectrophotometric determination of the filtrate showed 86% coupling of the IgG). 20 ml 1M ethanolamine was added to block any reactive sites. After 3 h at R.T., the gel was washed 3 times alternately with 100 ml 0-1M borate buffer (pH 8.0) containing 1M NaCl and 0.1M acetate buffer (pH 2.9) containing 1M NaCl. The gel was finally washed extensively with 0.05M phosphate buffered saline (PBS) (pH 7.5), and then poured into a 1x20 cm column.

The goat anti-mouse IgM serum was precipitated with 50% saturated ammonium sulphate; the resuspended pellet was dialysed extensively against 0.05M PBS (pH 7.5). The total protein content of this fraction was estimated (by OD_{280}) to be 10 mg/ml. When the column was equilibrated with the same buffer, 3 ml of the fraction was loaded on slowly to the column. The material not bound to the column (monitored automatically in a scanner by OD_{280}) was collected and the column was washed with 50 ml buffer. The bound protein was eluted with 0.1M glycine-HCl pH 2.9. The acidity of the eluate was neutralized as soon as the eluate dropped into tubes containing 0.5 ml 0.2 M borate buffer (pH 8.0). 1.0 ml eluate was collected in each tube. All

operations were done at $4^{O}C$. The eluted fractions were dialysed extensively against 0.05M PBS pH 7.5. The total yield of protein recovered was about 5 mg (OD_{280}); this purified antibody precipitated with normal mouse serum in Ouchterlony gels.

Purified goat anti-mouse IgA antibody was similarly prepared from antiserum raised against mouse intestinal sIgA, using a Sepharose 4B-IgA (MOPC 315) column; this was performed by Dr. D. Horsfall.

4.5.5 Rabbit anti-V.cholerae serum (RAB)

This was obtained from rabbits injected with 10^{9} log-phase live V.cholerae subcutaneously, weekly, for 4 weeks. The animal was bled from the ear 10 days after the last shot.

4.5.6 Rabbit anti-BSA serum

0.5 mg BSA in complete Freund's adjuvant was injected intramuscularly into a rabbit. Three weeks later, it was boosted with 0.5 mg BSA in saline i.m. Serum was obtained 10 days after the booster.

4.5.7 Mouse anti-V.cholerae intestinal juice (MAB#IIJ)

Mice orally immunized against BA or FLA (termed IMMUNE) were obtained in the following manner. The method of oral immunization with V.cholerae is described; that with S.adelaide was similar except that the bacterial dose used (both oral and i.p.) was 10-fold less.

Mice were fed with 5 doses of 10^{10} live log-phase V.cholerae in 20% $KHCO_3$ on alternate days. Two days after the last oral dose, the mice were injected i.p. with 10^7 live bacteria (or 1 µg BA). One week after this i.p. dose, the mice were used in absorption experiments or for obtaining immune intestinal juice. The latter was obtained by passing 1 ml cold PBS through the small intestine (see 4.4.4); the intestine was massaged gently but firmly as the fluid passed down. The wash-out was collected and centrifuged at 5,000g/15 min; the supernatant (termed IIJ) was kept frozen at $-20^{\circ}C$ until use. A partially purified extract was obtained by precipitation of the IIJ with 50% ammonium sulphate (see Table 7.1). Intestinal juice from nonimmunized mice (NIJ) was similarly obtained.

The activities of these antibody preparations were determined by the techniques described in the next section (4.6) and the results are listed in Table 4.2.

4.6   Detection of Antibodies

4.6.1   Bacterial agglutination

Glutaraldehyde-fixed bacteria were used here and in radioimmunassays (4.6.5). These were prepared from washed, log-phase bacteria. 0.5% (final) glutaraldehyde was added to the bacterial suspension (10^9 organisms/ml in saline). This was mixed occasionally for 15 min at room temperature. The cells were then washed with 5 x 50 vol saline (5,000g/15 min) and finally

Table 4.2   Characterization of some of the antisera and
immune intestinal juice (IIJ) used in the study

PREPARATION	T I T R E - 1			
	HA[@]	RIA[#]	FARR[*]	BACTERICIDAL
MAB#DIgM	640	7000	16	10^4
MAB#1276	3200	ND	96	10^6
MAB#IIJ	40-80	80	4	ND
Normal IJ	<20	20-40	<2	ND
RAB	800	ND	32	ND

ND = not done

[@] Passive haemagglutination

[#] Radioimmunoassay using I^{125} goat anti-mouse IgLC
     (titre expressed at 10% binding)

[*] Method of Minden & Farr (1973)
     (titre expressed at 10% binding)

resuspended in 2% BSA-PBS (pH 7.5) for use.

Two-fold falling dilutions of the antiserum were made in 2% BSA - PBS (50 μl) in microtitre plates (Medical Plastics) or in durham tubes. About 50 μl of the bacterial suspension (10^9 org/ml) was then added to each well with a standard dropper. The plate was incubated at 37°C/30 min and then overnight at 4°C before being read.

This technique was rarely used in the study because of its low sensitivity.

4.6.2   Passive haemagglutination (for protein antigens)

The procedure used for sensitizing red cells with protein antigens (BA, FLA and BSA) was modified from that of Hoq and Das (1970). In principle, the cells were fixed and tanned to make them sticky, though Beil et al (1972) reported that glutaraldehyde fixation alone was sufficient. This latter approach however, did not work with the above antigens.

Sheep red blood cells (SRBC) were washed three times in saline and then suspended in PBS (pH 7.5) as 5% (v/v). 2 ml 2.5% aqueous glutaraldehyde (Ajax) was added to 10 ml of the 5% SRBC. This was shaken constantly at room temperature for 15 min. The cells were then washed with 3 x 50 vol PBS, and finally made up as a 2% suspension in the same buffer. 20 ml of this 2% cell suspension was mixed with an equal volume of 1:80,000 tannic acid; this was incubated at 56°C/30 min with occassional shaking. The tanned cells were washed with 3 x 50 vol PBS and a 4% suspension was

then prepared in PBS. 1 vol of this suspension (usually 5 ml for each antigen) was mixed with an equal volume of antigen (20-40 µg/ml) in a capped test-tube; this was incubated on a roller at $37^{\circ}C$/45 min. The sensitized cells were again washed with 3 x 50 vol PBS and a 4% suspension was made in 2% BSA - PBS. These were all performed on the same day. (The efficiencies of sensitization for BA and FLA were 33% and 40% respectively, determined from the amount of antigen not bound to the cells by the inhibition of haemagglutination). The sensitized cells could keep for at least a month at $4^{\circ}C$ without significant loss of activity; they were well washed each time before use after storage for more than 2 days.

In the assay, the antiserum was titrated using a microtitre haemagglutination set (Cooke Engineering Co.), in 25 µl vol in 2% BSA - PBS. A drop (25 µl) of 0.125% sensitized cell suspension in the same buffer was added to each well. The plates were incubated overnight at $4^{\circ}C$ and then read.

Fig. 4.1 illustrates the sensitivity and reliability of the system. The sensitivity increased with decreasing cell concentration, at the expense of resolution. Thus, 4.8×10^5 cells/well (25 µl of 0.125% cell suspension) was used in all haemagglutination assays to achieve the highest sensitivity with reasonable resolution. (Other workers e.g. Hoq and Das 1970; Mahan and Copeland 1978, had generally used a ten-fold higher concentration of cells). About 1 ng anti-flagellin antibody could be detected by this assay (Fig. 3.3). The great sensitivity and simplicity of the system (more than a hundred antisera can be titrated in a day), and the fact that it can detect IgA antibodies

<u>Fig. 4.1</u>

<u>Passive haemagglutination using antigen-sensitized, glutaraldehyde-fixed,</u>
<u>tanned sheep red cells.</u>

ROWS A-E   used BA-sensitized cells (9.9 ng/4.8 x 10^5 cells).

ROWS F-H   used FLA-sensitized cells (4.6 ng/4.8 x 10^5 cells).

Diluting buffer used: PBS, pH 7.5, containing 2% BSA.

Results read after overnight incubation at 4^oC.

ROW A: Two-fold falling dilution of 2% sensitized cells made in
             0.05 ml buffer.
             0.05 ml buffer then added to each well.
             <u>Well 5</u> thus had 0.05 ml of 0.0625% cells or 4.8 x 10^5 cells.

ROW B: Duplicate of Row A

ROW C: As in Row A, except 0.05 ml of a dilution of specific antiserum
             added instead of buffer (see Row E).

ROW D: As in Row C, except half-strength antiserum used.

ROW E: Titration of mouse anti-BA serum using 4.8 x 10^5 sensitized
             cells/well.
             Thus , <u>Well 1</u> contained the same amount of antiserum and cells
             as in <u>Well 5, Row C</u>.

ROW F: Titration of purified mouse anti-flagellin using 4.8 x 10^5 cells.
             <u>Well 6</u> (end-point) contained 1.02 ng specific antibody.

ROWS G,   As in Row F, but done on the next day (in duplicate).
     H:

---
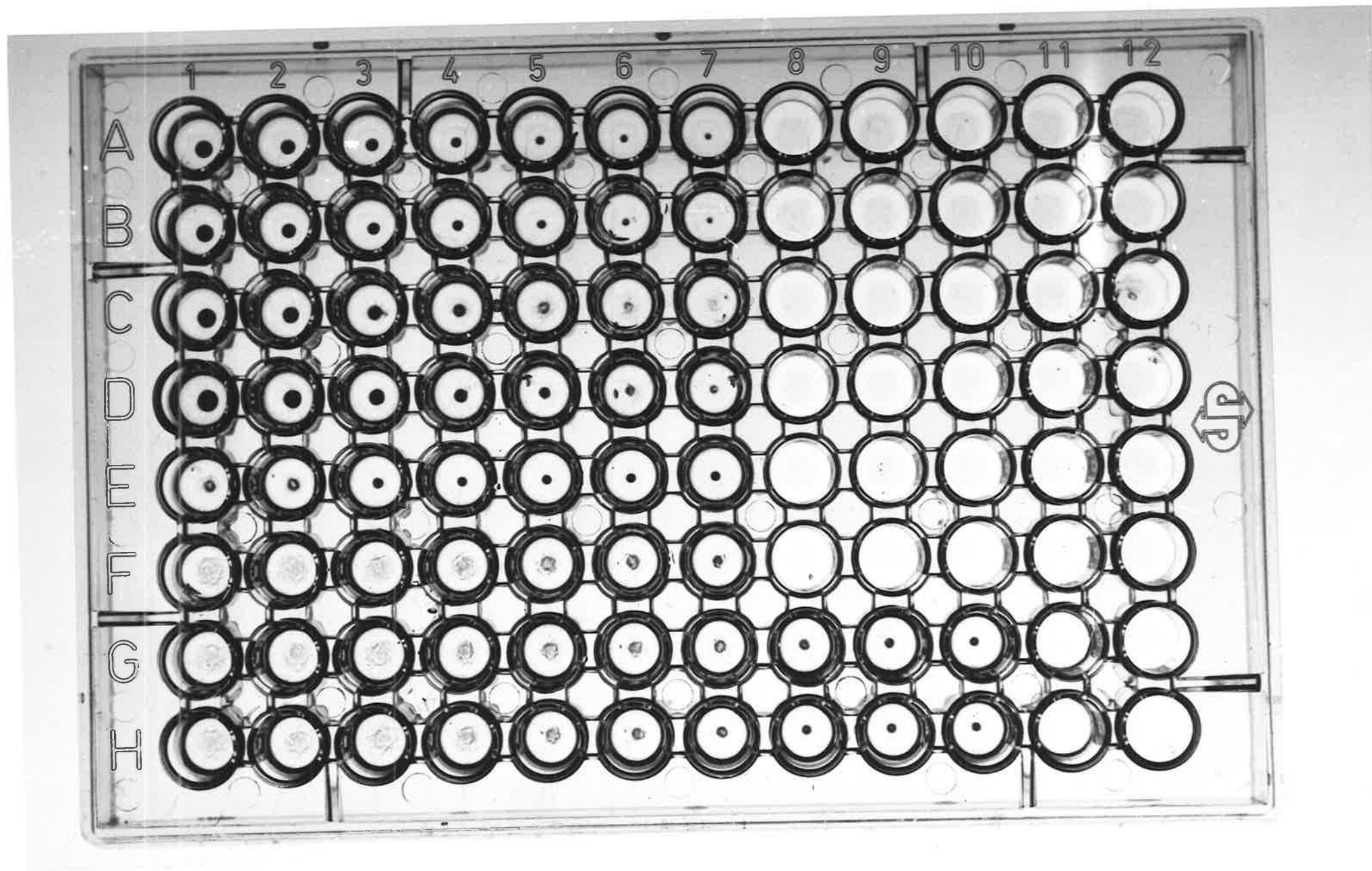
efficiently (see Fig. 4.2), make it a valuable tool.

4.6.3   Passive haemagglutination (for LPS)

The antigen (LPS) was initially treated with 0.02N NaOH, at a final concentration of 2 mg/ml in saline. After overnight stirring at room temperature, it was neutralized with 0.1N HCl.

A 2.5% SRBC suspension was made in saline from previously washed cells. The alkali-treated LPS was added to the cell suspension at a final concentration of 50 µg/ml in a capped test-tube. This was incubated at $37^{O}C$/90 min on a roller. The cells were washed with 3 x 50 vol saline and then made up as a 1% suspension in saline.

The haemagglutination assay was done in microtitre plates in 25 µl vol in saline, using 1% sensitized cells. The results were read after incubation at $37^{O}C$/45 min.

4.6.4   Farr assay

A micro-modification of the method of Minden and Farr (1973) was used. The mouse antiserum or intestinal juice was serially diluted in 0.05 ml PBS (pH 7.5) containing 2% normal mouse serum in 5 ml-serology tubes (Medical Plastics). 0.05 ml freshly dialysed I^{125}-antigen (about 10,000 cpm/10 ng) was then added to each tube. The tubes were mixed, sealed and incubated overnight at $4^{O}C$. 0.1 ml cold saturated ammonium sulphate (87% sat solution used for some antigens e.g. BSA) was added to each tube; this was mixed immediately and vigorously in a vortex. The tubes were sealed and incubated overnight at $4^{O}C$ again. The

Fig. 4.2

Binding curves of MOPC315 and rabbit anti-BSA serum using the
Farr assay.

Method as described in 4.6.4 using 4000 cpm (1 μg) I^{125}-DNP$_5$-BSA
and 87% sat. ammonium sulphate.

(The haemagglutination titre ($\downarrow$) of MOPC315 was determined previously
 as detailed in Fig. 4.4; that of rabbit anti-BSA was determined
 using DNP-BSA sensitized SRBC.)

■   MOPC315

□   rabbit anti-BSA serum

Fig. 4·2
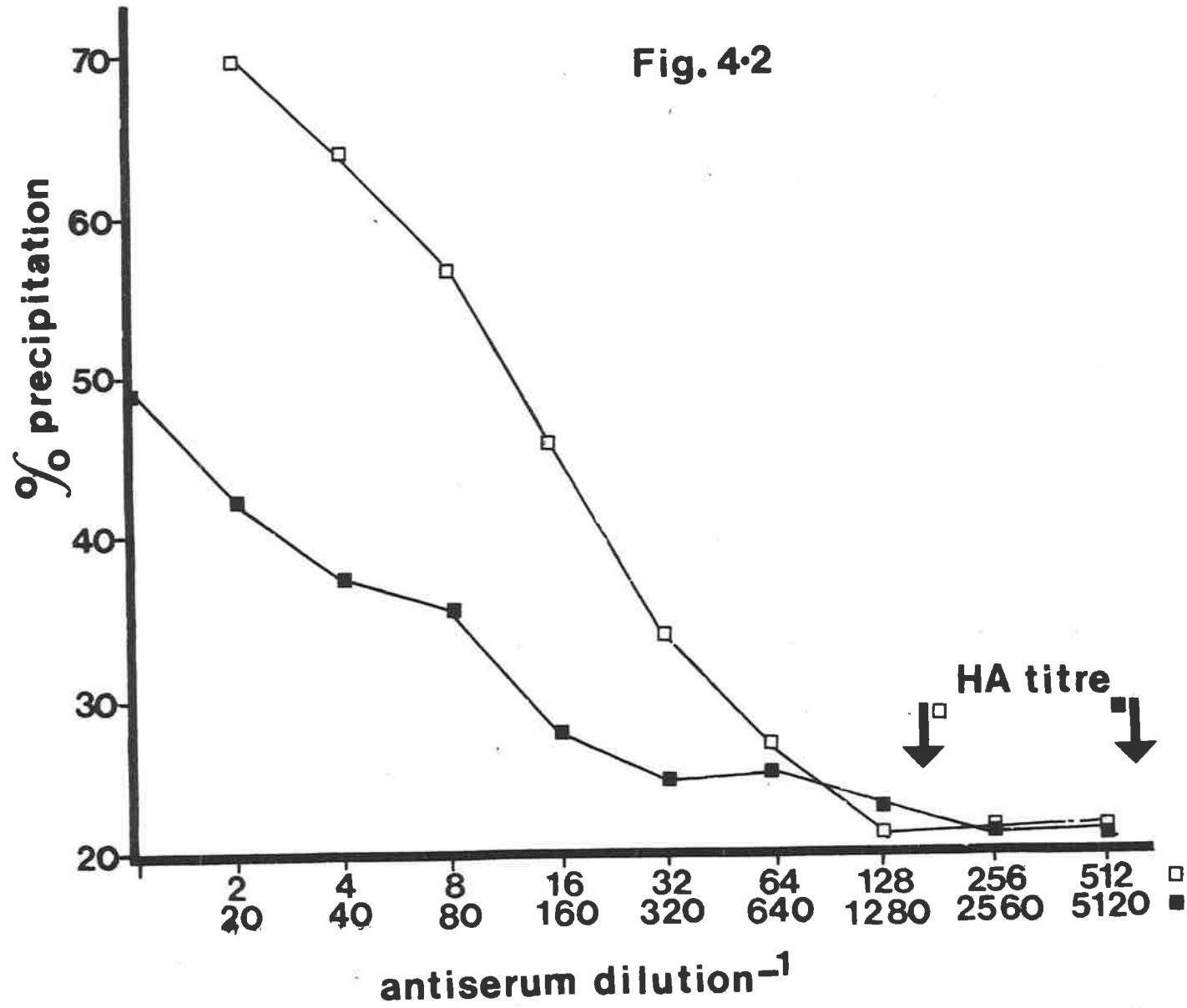
precipitate obtained by centrifugation at 2,000g/20min was washed twice with cold 50% sat ammonium sulphate and then counted.

Fig. 4.2 shows the results of this assay for 2 antibody systems (rabbit anti-BSA and mouse myeloma (MOPC 315) anti-DNP) in comparison with their haemagglutination titres. The Farr assay was less sensitive in these two systems (expressing the titre at the 33% binding level) and consequently it was seldom used to detect antibodies in the study. However, it was used to demonstrate the antigenicity of radiolabelled compounds absorbed from the intestine (see 4.8.4 and Chapter 6).

4.6.5   Double antibody radioimmunoassay (RIA)

This technique makes use of a second antibody (labelled with I^{125}) to detect primary antibodies bound to an insoluble immunoabsorbent, similar to that described by Robertson and Cebra (1976), and the solid phase system of Zollinger et al 1976. The insoluble antigen matrix used was either glutaraldehyde-fixed bacteria (4.6.1) or antigen-sensitized fixed SRBC (4.6.2).

The mouse antiserum or intestinal juice was titrated in 2-fold falling dilutions in 0.05 ml 2% BSA - PBS (pH7.5) in 5 ml-serology tubes (Medical Plastics). About 0.05 ml of immunoabsorbent (washed and suspended in 2% BSA - PBS at 10^{11} org/ml) was added to each tube with a graduated pasteur pipette. This was mixed in a vortex and incubated overnight at $4^{\circ}C$. The haemagglutination or agglutination titre was noted. The cells were then washed twice with 2 ml 0.5% BSA - PBS (pH 7.5) (2,000g /20 min) and the washed pellet was resuspended in a drop (about 0.05 ml) of I^{125}

Fig. 4.3

Binding curves of mouse anti-*V. cholerae* serum (MAB#DIgM) using
V. cholerae variously treated.

Method as described in 4.6.5 using 10^8 *V. cholerae* treated in various
ways prior to assay, and 5000 cpm (5 ng) I^{125}-goat anti-mouse IgLC.

Status of bacteria

O   live

□   glutaraldehyde-fixed

●   steamed/30 min

■   treated with 20 vol   ethanol 56^0/30 min

Fig. 4·3
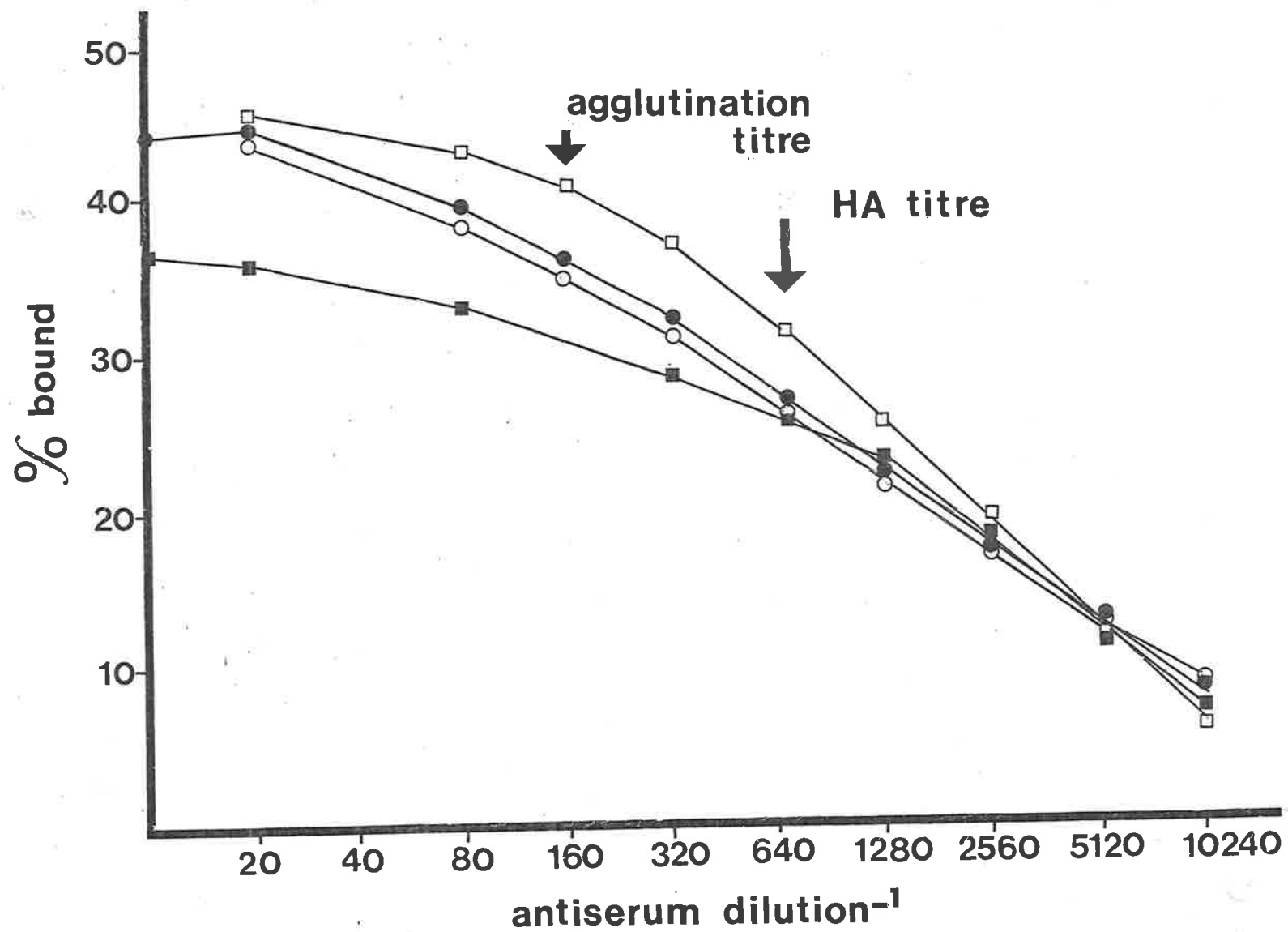
goat anti-mouse Ig (in 2% BSA - PBS; about 200,000 cpm/ml) delivered from a pasteur pipette. This was mixed and incubated for 1 - 24 h at 4°C. The cells were again washed twice with 2 ml 0.5% BSA - PBS and then counted.

The results of a typical assay using V.cholerae variously treated as the immunoabsorbent are shown in Fig. 4.3. The radioimmunoassay titre (expressed at 10% binding) for this serum (MAB # DIgM) was about 10-fold higher than that obtained by agglutination, but the assay was only marginally more sensitive than passive haemagglutination. This latter observation was also apparent with the FLA anti-FLA system (Fig. 3.3). In fact, the radioimmunoassay was less sensitive than haemagglutination in detecting IgA anti-DNP antibody (MOPC 315) using DNP_5-BA-SRBC as the immunoabsorbent (Fig.4.4); the radioimmunoassay was developed with both I^{125} goat anti-mouse LC and I^{125} goat anti-mouse IgA. Since the radioimmunoassay profile for this antibody (MOPC 315) is similar to that obtained by the Farr assay (Fig. 4.2), which suggests a low-avid antibody (which in fact has an association constant of 2.0 x 10^6 M^{-1}; Pecht et al 1972), both assays may not detect low-avid antibodies efficiently. (See Chapter 9 and Celis et al (1977) on this point.) Thus, in some studies (Chapter 8), the radioimmunoassay was merely expressed as the total amount of labelled goat antibody bound per volume of mouse antiserum used (instead of as a titre). The fact that the radioimmunoassay is not subjective makes it a useful confirmatory test of haemagglutination. In practice, it was often done after the haemagglutination on the same reacted cells, without any waste of material. More immunoabsorbent (bacteria) was added to each well to increase the resolution; after overnight incubation at

Fig. 4.4

Binding curves of MOPC315 using the double antibody radioimmunoassay.

Different amounts of MOPC315 were reacted with DNP_5-BSA sensitized SRBC (0.2%, final) for 18 h/4°C. The haemagglutination titre was noted. The cells were then washed twice and reacted either with I^{125}- goat anti-mouse sIgA (□) or I^{125}- anti-mouse IgLC (○).[*] After incubation overnight/4°C, the cells were washed twice and counted.

*(The specific activities of both preparations were about 1000 cpm/ng, and 3000 cpm/tube were used in both.)

Fig. 4.4
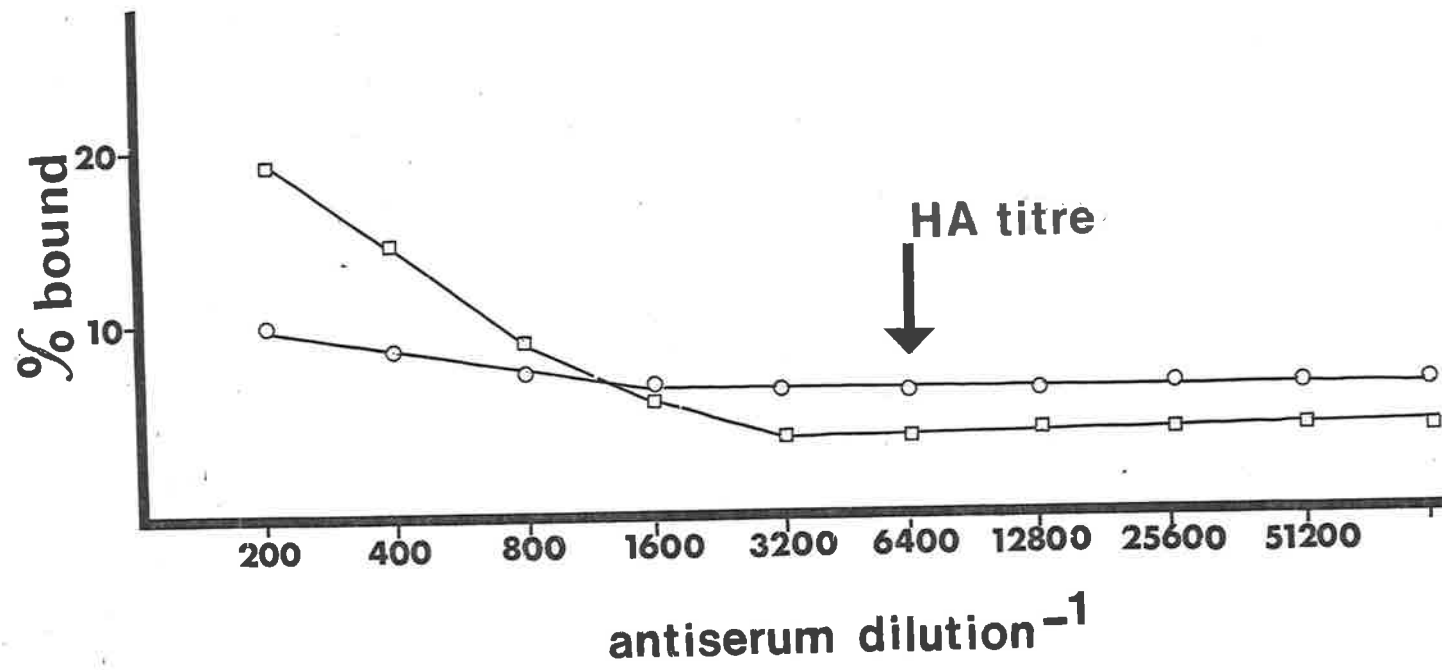
4°C, the cells were washed in 5 ml-serology tubes and the assay was performed as before.

4.6.6   Opsonic assay

See Chapter 8.

4.7   Techniques in Animal Experimentation

4.7.1   Oral administration in the mouse

Mice were fed using a 1 ml syringe and a 19G solder-tipped needle. The mouse was held firmly by the neck between the thumb and second finger, and the head was tilted back by the first finger while the needle was introduced into the oesophagus.

4.7.2   Oral administration in the rat

The rat was anaesthetized with ether, and the antigen was administered into the stomach through a soft catheter tubing (as described by Hemmings and Williams 1978).

4.7.3   Obtaining serum/plasma from the mouse

Mice were bled by the retro-orbital plexus with a pasteur pipette. Serum was obtained by allowing the blood to clot at 37°C/30 min, followed by overnight incubation at 4°C (for better contraction of the clot). Plasma was obtained e.g. in 6.3.1 by mixing 0.13 ml blood with 0.15 ml Hanks medium containing 10 U/ml

heparin, from which 0.175 ml plasma was obtained (with a Finn pipette) after low-speed centrifugation.

4.7.4   Obtaining serum from the rat

The thorax of the anaesthetized rat was reflected, and blood was withdrawn from the heart using a 19G needle and a 20 ml syringe. The blood was clotted at $37^{\circ}C$ /1 h and serum was obtained after a further incubation of 2 h at $4^{\circ}C$.

4.7.5   Thoracic cannulation in the mouse

The starved mouse was fed with 0.5 ml cream. After 1 h, it was anaesthetized (with Avertin), and pinned down on its right side. A small incision was made on the left side just below the diaphragm. The thoracic duct was located and a 12" BOAK cannula (Portex, Hythe Kent) was inserted and fixed. The cavity was closed, and the animal was allowed to recover in the warmth of a lamp. Just before it had recovered, it was clamped firmly by the abdomen onto a rotatable drum. It was given food and water, and lymph was collected from the cannula. (The guidance of Dr. C. Jenkin in this technique is acknowledged).

4.7.6   Liver perfusion in the mouse and rat

After the animal was killed, the liver was exposed. A 12" BOAK cannula (Portex, Hythe Kent) was inserted into the inferior vena cava (at the top of the liver). Saline contained in a 10 ml syringe connected to the cannula was perfused through the liver till the organ turned pale.

4.7.7   Studies with the mouse small intestine

To obtain the small intestine of the mouse, the animal was killed and the abdomen reflected. The mesenteric lymph nodes were located and excised, so that the small intestine became loose. The intestine was cut 1 cm below the pyrolus and 1 cm before the ileal-caecal junction, and this length is considered the whole small intestine. It was placed on ice as soon as it was obtained.

In experiments where the intestinal contents were required, e.g. for counting the amount of I^{125} antigen (7.3), the contents were flushed out and collected with 1-3 ml saline delivered from a feeding needle and syringe. In others where the washed intestine was required e.g. for determining the amount of labelled antigen adsorbed (7.2), the intestine was connected to a tap (through a modified Finn-pipette tip) and a slow jet of water was passed through it for about 0.5 min. The washed intestine was sometimes segmented into 1 cm long pieces (6.1.1a) or into Peyer's patches (Pp) and non-Peyer's patch (nP) tissue (6.2 and Chapter 8). In the latter, the Pp were located on the serosal side, and these were carefully excised with a pair of scissors so that as little as nP tissue was contained. An equivalent mass of nP tissue immediately adjacent to the Pp was also carefully excised. The Pp (or nP) were always pooled for the same intestine and these were also pooled for the same group of mice in some studies e.g. 8.4.

4.7.8   The everted gut sac model

The gut sac model used here was modified from those of Walker et

al (1972a) and Nolan et al (1977). The unwashed small intestine (see 4.7.7) was segmented in the middle into the jejunum (proximal half) and the ileum (distal half). The jejunum was ligated (with a thread) 1 cm from the proximal end while the ileum was ligated 1 cm from the distal end. 11 cm long sacs were made from the ligated ends in the following way. The intestine was carefully everted over a small metal rod (1 mm diameter) at the ligature. After the everted intestine was cut to the desired length, a feeding needle was tied to its free end. 0.5 ml Hanks medium was introduced into the sac with a syringe (inserted into the feeding needle) and any sac that leaked was discarded. The filled sac was placed in a 10 ml (centrifuge) tube containing 2 ml Hanks medium and the I^{125} antigen. While the sac was completely immersed in the bath, the feeding needle was suspended above it by a thread. The tube was aerated with excess oxygen and then sealed. It was incubated at $37^{\circ}C$ for 1 h (unless otherwise stated), after which it was taken out and rinsed gently in 4 successive baths of 10 ml Hanks medium. The sac contents were carefully withdrawn with a 1 ml syringe inserted into the feeding needle while the other end of the sac was hooked on to a support. The sac was rinsed twice with 0.5 ml Hanks medium and these wash-outs were pooled with the sac contents. This was counted, dialysed and counted again. (In some experiments, the everted intestine was treated with 1mM dithiothreitol (Sigma) in Hanks medium at $37^{\circ}C/15$ min (Bull and Brookman 1977) and then rinsed thoroughly in the same medium before the sacs were made).

4.7.9   Tissue processing

The excised organ (liver, spleen, lymph node, kidney, skeletal

muscle or intestine) was homogenised in a small volume of saline in an ultra-turrax (Janke & Kunkel, KG Ika Werk, Staufen i. Breisgan, Germany). Samples of the homogenate were counted, dialysed and counted again (e.g. 5.3 and 6.2). In the extraction of $H^3DNP-BA/BA$ from tissue, the homogenate was frozen and thawed twice, ultrasonicated (1 min), treated with 0.5M TCA, dialysed and centrifuged (10,000g/20 min). The supernatant obtained was concentrated (using aquacide), dialysed, counted and used for priming (Chapter 8), while the precipitate was lyophilized and counted (6.2). (If necessary, all organs were stored at -20°C until processed.)

4.8   Miscellaneous Immunochemical Techniques

4.8.1   Immunodiffusion in gel

The technique as described by Ouchterlony (1973) was used.

4.8.2   Protein estimation

The Folin-Ciocalteu reaction, as described by Williams and Chase (1968) was used.

4.8.3   Carbohydrate estimation

The phenol-sulphuric acid method, as described by Williams and Chase (1968) was used.

4.8.4   <u>Antigenicity test of radiolabelled antigens</u>

The Farr assay (4.6.4) was used to detect specific antigens. 0.05 ml of a strong precipitating antiserum (MAB # 1276; MAF) and 0.05 ml of the label were incubated before precipitating with 50% sat ammonium sulphate. (Standards of the native antigens in serum were included.)

4.8.5   <u>Antigenicity test of I^{125} labelled mouse IgG</u>

The procedure is similar to 4.8.4 except that purified goat anti-mouse IgLC and 3.7% polyethylene glycol (instead of ammonium sulphate; Digeon <u>et al</u> 1977) were used.

4.9   <u>Others</u>

Specific procedures with some experiments are given in the relevant sections in the experimental chapters. Buffers e.g. phosphate-buffered saline (PBS), Hanks medium, etc. are as described by Williams & Chase (1968) and Hudson & Hay (1976).

4.10   <u>Statistics</u>

The Mann-Whitney rank test programmed in the University computer, was used for all results. (See Denenberg 1978; Campbell 1974). The results are usually expressed as mean $\pm$ S.E.

5.   CHAPTER 5. THE PREPARATION AND CHARACTERISATION OF THE

ANTIGENS

The majority of studies on the intestinal absorption of
macromolecules (e.g. Warshaw et al 1974; Andre et al 1974) have
used serum albumin (human or bovine) as the antigen. The reason
often advanced for this choice is its clinical relevance as a
dietary protein. Its availability in purified form commercially
is undoubtedly an important consideration. The absorption of
antigens from other sources, or of a different chemical
constitution, has received less attention. For instance, there
has been no study on the absorption of flagellin, despite the
intensive research on its immunogenicity and tolerogenicity
systemically (Parish and Ada 1972). For this reason and others
(mentioned in 2.2), we are interested in two bacterial antigens :
Boivin Antigen(BA) extracted from V.cholerae 569B, and flagellin
(FLA) derived from S.adelaide. (The possible role of the former
in gut-derived endotoxaemia is also relevant). The preparations
of BA and FLA are described in this chapter. The antigens are
also characterized according to their chemical nature and their
stability in biological situations, and hence, their suitability
in oral absorption studies.

5.1   Preparation of BA

The preparation of BA is based on the trichloroacetic acid
(TCA)-extraction method of Boivin, as described by Kabat (1976b)
and Williams and Chase (1967).

Bacteria (V.cholerae 569B) grown in nutrient broth shaken in 2-litre flasks at 37°C for 4 h were usually used. The washed bacteria were resuspended in saline at a concentration of 10^{10} organisms/ml, and kept chilled. An equal volume of cold 0.5 M TCA was added to the suspension. This was stirred overnight at 4°C. The extracted bacteria were centrifuged at 5,000g/30 min. The supernatant was clarified and concentrated if necessary. It was adjusted to pH 6.5 with 20M NaOH, chilled in ice and poured into 2 volumes of ethanol kept chilled in an alcohol-ice bath; this was left overnight in the cold room to precipitate. The precipitate obtained after centrifugation at 5,000g/30 min/4°C was dissolved in a small volume of distilled water and dialysed extensively in distilled water. It was clarified off any debris (5,000g/15 min) and then centrifuged at 70,000g/2 h. The pellet obtained was dissolved in a small volume of deionized, distilled water - this is the crude BA preparation.

Attempts were made to purify the antigen further. Galanos and Luderitz (1975) obtained a homogenous preparation of LPS by electrodialysis, which reportedly removes metal cations and basic amines from the crude extract. This method was used in an experimental set-up, which consisted of a bent glass-tube filled with BA, and sealed at both ends with dialysis tubing. The ends were immersed into separate beakers of distilled water. An electric circuit was connected with one end as the anode and the other as the cathode. After 24 hours, the material that precipitated at the anode was collected and examined. It was unfortunately, as heterogenous as the crude preparation when fractionated on Sephadex G200.

Another approach was used to obtain a more stable and homogenous preparation of BA. The crude preparation (1gm) was extensively digested with trypsin (10mg; BDH) at 37°C for 5 days in 0.15M phosphate buffer (pH 8.2), following a procedure described by Freeman et al (1940). The digest was clarified (5,000g/15 min) and the pH of the supernatant was adjusted to 6.5. When chilled, it was added to 2 vol (400 ml) of ethanol and allowed to precipitate overnight in the cold. The precipitate was dissolved in distilled water and fractionated in an Ultrogel column (AcA22; LKB), in 0.5M glycine-NaOH buffer (pH 10.0), containing 0.75% sodium deoxycholate (BDH). A typical fractionation profile is shown in Fig. 5.1. The different pooled fractions (I - IV) were obtained. The pH was adjusted to 6.5 and each fraction was precipitated with 2 vol of ethanol as before. The precipitates were resuspended in water and dialysed (sodium deoxycholate is soluble in ethanol at that pH).

The preparation from Fraction IV (BA #abc) was used for all radiolabelling procedures in the study. As would be expected, it has a low protein content compared with other preparations (Table 5.1). It could nevertheless be iodinated readily - see Table 4.1. (Ideally, all the different preparations should be pooled, but this was not possible as these were made at different times.)

5.2   Preparation of FLA

The method of preparing FLA was derived from a combination of sources (Nossal and Ada 1964; Kobayashi et al 1959; Williams and

Fig. 5.1

Fractionation of crude BA#abc on Ultrogel AcA22 (3 x 100 cm)
in 0.5M glycine-NaOH (pH 10.0) containing 0.75% sodium deoxycholate.

Figures indicate recovery (% dry weight, total mass) in each
fraction (I - IV).

Fig. 5·1
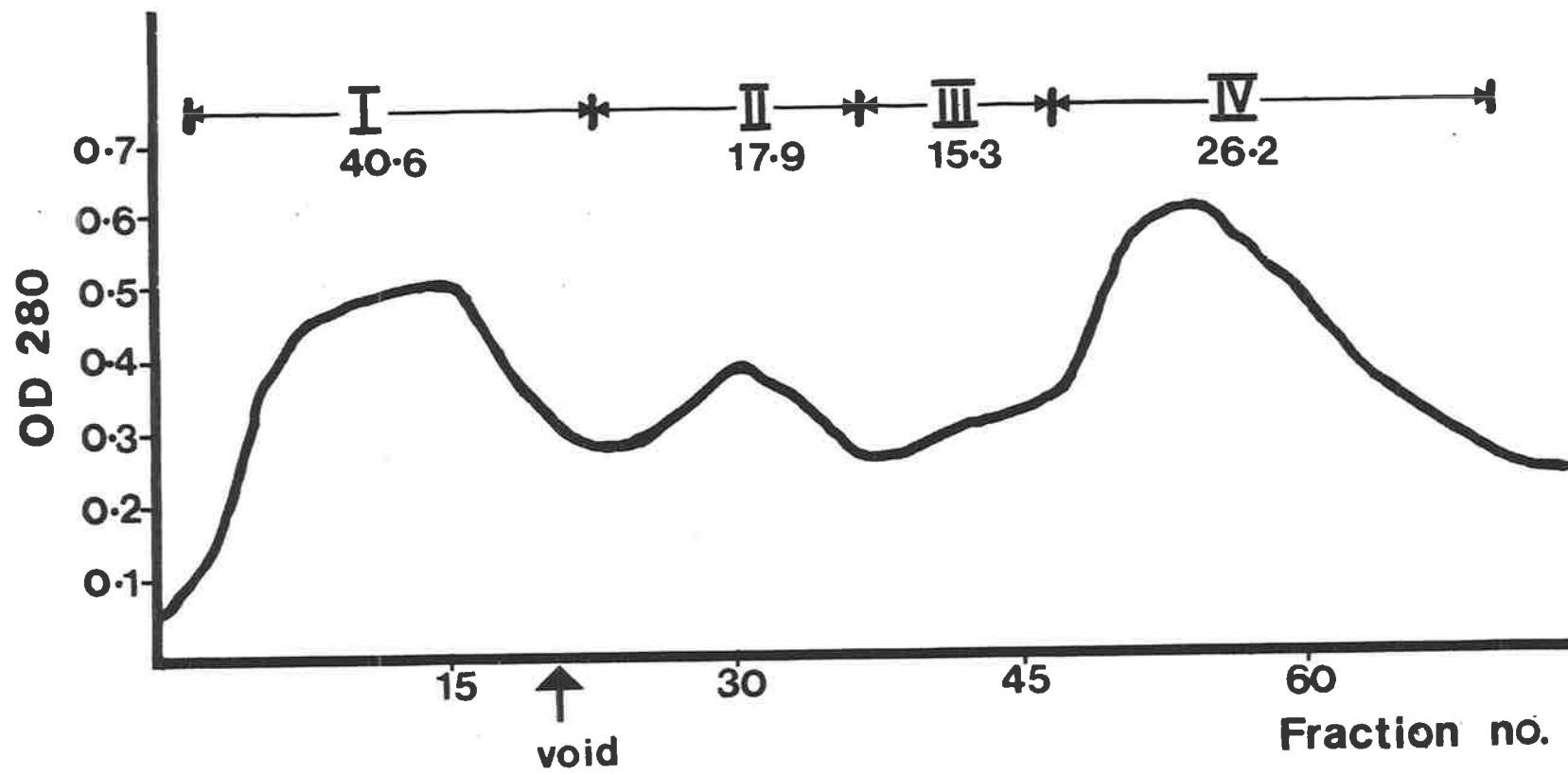
Table 5.1   Characteristics of BA preparations

BA prep	dry wt[*] (mg/ml)	% CHO[@]	% protein[#]	CHO:protein	%recovery[+]
DBA	3.8	6.6	17.9	0.37	ND
abc	2.5	78.0	3.2	26.00	ND
578	20.0	48.0	33.9	1.42	1.6
678	14.0	53.1	29.9	1.78	2.7
878	15.0	17.3	37.6	0.46	ND
179	6.5	17.7	31.9	0.56	ND

ND = not determined

[*] By dehydration of 0.1 - 0.2 ml samples

[@] Carbohydrate estimation by phenol-sulphuric acid method

[#] Method of Folin - Ciocalteu

[+] Yield of BA (dry wt.) / yield of bacteria (dry wt.)

Chase 1967; Dimmit and Simon 1971).

S.adelaide bacteria were grown on solid nutrient agar in large plates (30 x 30cm) at $37^{\circ}C$ in a humid atmosphere. After 24 hours, the bacteria were harvested, washed once in saline, and suspended in saline at a concentration of 10^{10} org/ml. Motility of the organisms was checked microscopically. The suspension was blended vigorously for 1.25 min (ATOMIX, MSE, London; half-speed) and centrifuged at 10,000g/20 min. The supernatant obtained was centrifuged at 48,000g/1 h. The resulting pellet was dissolved in 50 ml 0.015M PBS (pH7.5) and then dialysed against the same buffer for 2 h/$4^{\circ}C$. The solution was finally clarified (10,000g/15 min). This is termed the crude preparation. It was further purified thus.

It was applied to a DEAE cellulose-52 column (3 x 30 cm), which was previously equilibrated with the same buffer. Most of the protein was bound to the column (monitored by OD_{280}). It was eluted with 0.25M NaCl and 0.5M NaCl in the same buffer. Each eluate was precipitated with 0.33 vol cold saturated ammonium sulphate at $4^{\circ}C$; this was stirred gently for 2 hours and then left to precipitate overnight. Electronmicroscopic examination of the dialysed precipitate showed that the 0.25M eluate had very good, intact flagellae while the 0.5M eluate also had good flagellae but were more fragmented. Both preparations were combined and fractionated on a sucrose density gradient (a technique not used previously). This consisted of 5 ml 58% sucrose and 8 ml 55% sucrose (derived from preliminary studies). 17 ml of the flagella preparation was carefully layered on the top of each gradient; this was centrifuged at 55,000g/4 h (rotor

SW25.1). A pellet was formed and a whitish material was distributed throughout the two sucrose layers. These layers were separately collected and centrifuged at 48,000g/1 h; a clear, purplish-blue pellet was formed in both. Pure, homogenous flagellae were observed in these pellets (Fig. 5.2), while the pellet obtained from the sucrose density gradient showed good flagellae mixed with contaminating vesicles. This latter preparation was re-extracted together with the supernatant of the ammonium precipitation. They were centrifuged at 48,000g/1 h, and then fractionated on the sucrose density gradient as described. The fractions were centrifuged at 48,000g/1 h again, and all fractions showing pure flagellae (by electronmicroscopy) were pooled. This is termed the pure flagella preparation. The crude and pure preparations were characterised as shown in Table 5.2.

Flagellin was obtained by acid dissociation (Abram and Koffler 1964). 0.1 ml 1N HCl was added to 2 ml flagella (2 mg/ml). This was incubated at room temperature for 30 min. It was centrifuged at 32,000g/90 min. A small pellet was obtained and discarded. The supernatant was neutralized with 1N NaoH or dialysed against distilled water. This is the flagellin preparation (FLA) which was used in all studies (red cell sensitization, radiolabelling, etc.) except in oral feeding, where purified flagella was used. It has no detectable LPS (by the inhibition of haemagglutination assay). It was pure on denaturing (SDS) polyacrylamide gel (Lugtenberg et al 1975) compared with the crude flagella, and showed a mol. wt. of 50,000. (This was kindly performed by Mr. A. Richardson.)

Fig. 5.2

Electronmicrograph of purified flagella from *S. adelaide*.

X 45,000;   phosphotungstic acid stain.
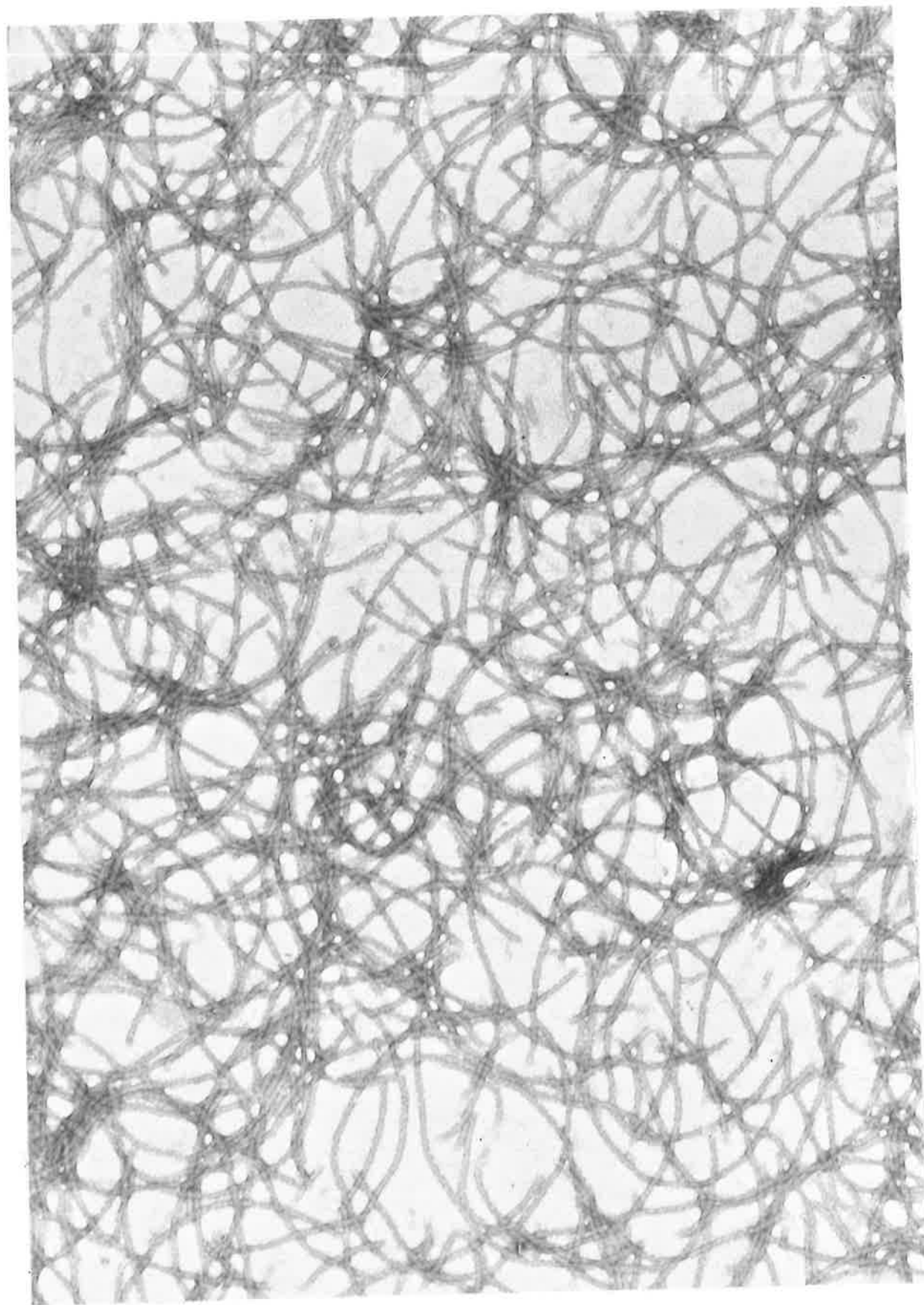
Table 5.2   Preparation of S.adelaide flagella

Total dry wt of bacteria used = 36.6 gm

	CRUDE PREPARATION	PURIFIED FLAGELLA
TOTAL YIELD[*]		
- dry wt (mg)	533	137
- % dry wt bacteria	1.46	0.37
% PROTEIN[#]	76.9	95.0
% LIPOPOLYSACCHARIDE[@]	14.2	0.02

[*] from lyophilized mass (PURIFIED FLAGELLA) or dehydration
    of 0.1 ml sample (CRUDE PREPARATION)

[#] by method of Folin-Ciocalteu

[@] by inhibition of passive haemagglutination

5.3   The Behaviour of the Antigens in vivo

The fates of the two antigens, BA and FLA, are quite different in the mouse. Thus, when I^{125} BA and I^{125} FLA were injected intravenously by the femoral vein in mice, the latter was rapidly cleared from the circulation and tissues, while BA was less readily removed (Fig. 5.3). This was determined from the total radioactivity recovered in the blood (0.13 ml), liver, kidney and spleen between 5 min and 2 hours after injection. With FLA, there was a high uptake of the antigen by the liver (27% of dose given) at 5 min post injection. However, the liver-associated label fell rapidly so that only 3% of dose administered remained after 2 hours. This was probably due to degradation of the antigen in situ, as the amount of labelled material of high molecular weight in the liver (estimated by dialysis of tissue homogenate) also declined from 68% (of associated radioactivity) at 5 min to 45% at 30 min. In contrast, there was a lower uptake of BA by the liver (15% of that administered), and this remained fairly constant throughout the study. Moreover, the % non-dialysability of the radioactivity in the liver homogenate here also remained high throughout (about 80%). In other words, unlike FLA, BA was not readily removed or degraded by the liver. As such, an appreciable amount of antigen persisted in the circulation (4-5% dose/0.13 ml blood) during the 2 hour-period. On the other hand, very little FLA could be detected in the blood even at 5 min post injection (3%); there was less than 1% remaining at the end of the study. The uptake of these two antigens by the kidneys paralleled their clearance rates from the circulation; this probably reflects blood-borne radioactivity. The spleen, on the other hand, was very similar to the liver in the absorbance of these antigens.

Fig. 5.3

Recovery of *i.v.* injected, I^{125}-labelled antigens in mice.

Following the *i.v.* administration of (A) I^{125}-FLA (72,000 cpm; 90 ng)
and (B) I^{125}-BA (112,600 cpm; 176 ng) to 2 mice in each group, the
undialysed radioactivity present in the blood and different organs was
determined at various times. This is expressed as a % of the dose given;
the numerical values shown indicate the % non-dialysability of the radio-
activity present in liver extracts.

▲   liver (unperfused)

□   kidney

○   spleen

●   blood (0.13 ml)

Fig. 5.3
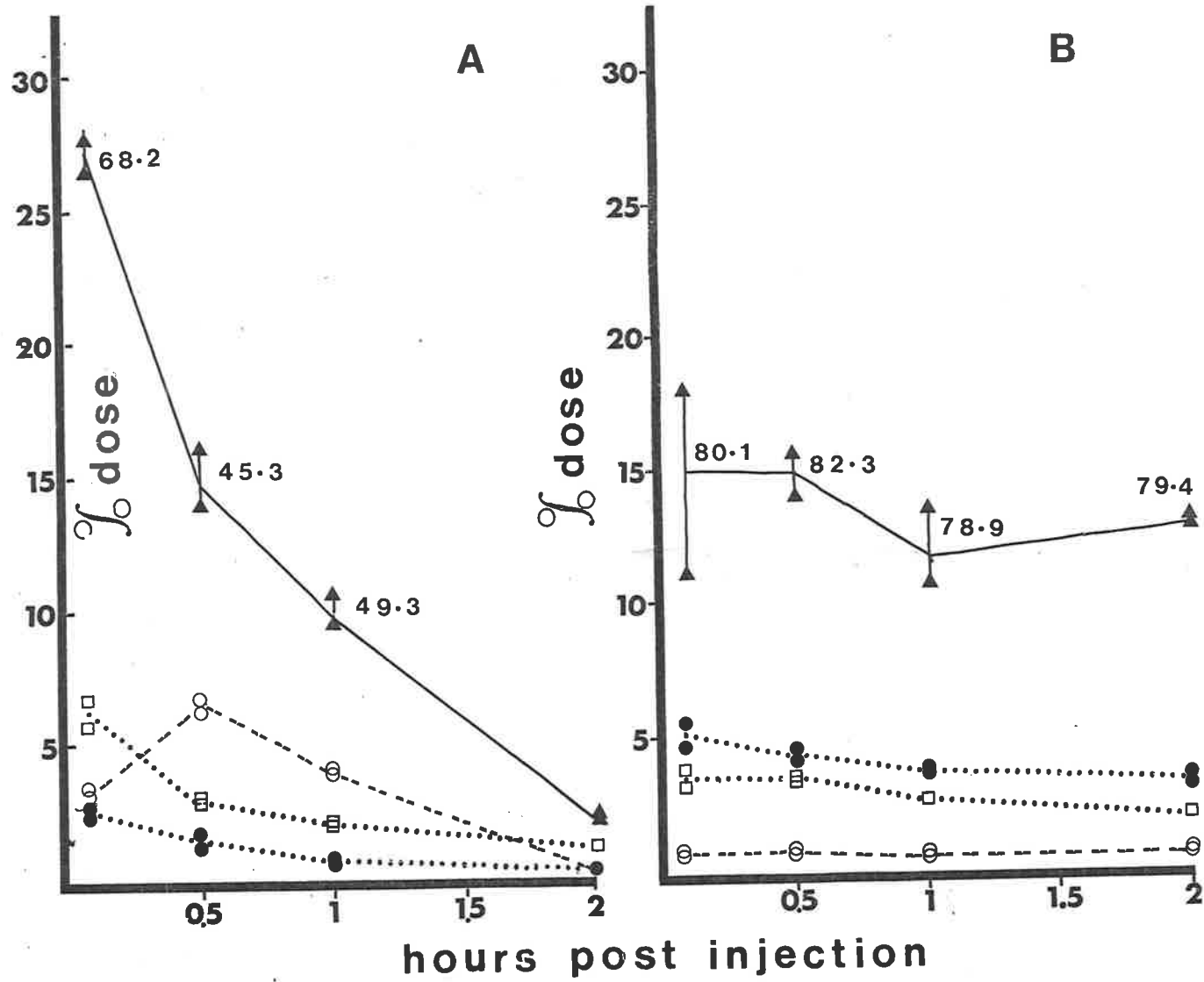
It is therefore not surprising that no antigen was detected in the serum of normal mice injected i.v. with cold FLA at various times post injection, using the inhibition of haemagglutination assay (3.1.1); however, antigen was detected if the liver function was blockaded with carbon (method of Jenkin & Rowley 1961) (Table 5.3). In contrast, as much antigenic BA could be recovered from the serum of normal mice as that from carbon-blockaded mice using a similar approach (Table 5.4). Further, the liver uptake of BA could be enhanced by pretreatment of the antigen with specific antibodies; this enhancement could again be abolished with carbon (Table 5.5).

5.4 The Stability of the Antigens in Mouse Intestinal Juice

The degradation of BA and FLA by normal mouse intestinal juice (NIJ) was examined, as this is pertinent to the oral absorption of these antigens. I^{125} BA, I^{125} FLA and I^{125} BSA were incubated with NIJ at $37^{\circ}C/90$ min. Samples of the digests were fractionated on Sephadex G50. It is apparent from Fig. 5.4 that while 95% of FLA (based on total radioactivity) was degraded to low molecular weight forms, BA was relatively stable. 60% of the radioactivity in BA was excluded in the column; the loss of label here may merely be due to deiodination rather than degradation. BSA, for comparison, was intermediate between the two in terms of stability under these conditions.

5.5 Discussion

The bacterial antigens, BA and FLA, were prepared and

Table 5.3   Recovery of i.v.-injected FLA from mouse serum[*]

Time after injection (min)	NORMAL MICE 2-fold Serum dilution					BLOCKADED MICE[@] 2-fold Serum dilution					
	1	2	3	4	5	1	2	3	4	5	6
0	+	+	+	+	+	ND	−	−	−	−	+
10	+	+	+	+	+	ND	−	−	−	+	+
20	+	+	+	+	+	ND	−	−	−	+	+
30	ND	+	+	+	+	ND	ND	ND	ND	+	+

ND = not done;   + = haemagglutinated;   − = inhibited reaction

[*]   10 µg FLA was injected i.v. and the serum obtained at various times thereafter was titrated for FLA by the inhibition of haemagglutination. First well contained 50 µl test serum & 150 µl buffer

[@]   Injected i.v. with 2.4 mg carbon 15 min prior to exp.

(Results averaged for 3 mice / group)

Table 5.4   Recovery of i.v.- injected BA from mouse serum[*]

Time after injection (min)	NORMAL MICE 2-fold serum dilution					BLOCKADED MICE[@] 2-fold serum dilution				
	1	2	3	4	5	1	2	3	4	5
0	−	−	−	+	+	−	−	−	+	+
6	−	−	−	+	+	−	−	−	+	+
14	−	−	−	+	+	−	−	+	+	+
20	−	−	−	+	+	−	−	+	+	+
35	−	−	+	+	+	−	−	+	+	+

[*]   As in Table 5.3 except that 555 µg BA#DBA was injected and the first well contained 5 µl serum & 195 µl buffer

Table 5.5   Effect of carbon blockade on the hepatic uptake
of I^{125}BA-antibody complexes

Status of mice	NORMAL	NORMAL	BLOCKADED[#]	BLOCKADED[#]
Ag treatment[*]	BA	BA-Ab	BA-Ab	BA
Liver uptake[@]	11.4±0.2	16.8±0.7	9.52±0.90	10.1±1.0
Kidney uptake[@]	2.87±0.40	2.20±0.08	4.57±0.88	3.50±0.05

[#]   injected i.v. with 8 mg carbon 1.5 h prior to exp.

[*]   injected i.v. with ca.60,000 cpm (0.1 µg) I^{125}BA mixed
with 20 µl normal mouse serum (BA) or 20 µl mouse anti-BA
serum # DIgM (BA-Ab)

[@]   Total radioactivity in liver (unperfused) or kidney
30 min post injection, expressed as % dose given ±S.E.
(3 mice / group)

Fig. 5.4

Fractionation of I^{125}-antigens digested with normal mouse intestinal juice on Sephadex G50 (column size: 1 x 27 cm).

Equivalent amounts of intestinal juice were used for all antigens. About 0.05 ml I^{125}-antigen (150,000 cpm/200 ng) was incubated with 1 ml fresh normal mouse intestinal juice at 37^{0}C/90 min . The excluded material (fractions 1 - 6) represents 60.7%, 41.5% and 4.9% of the total radioactivity in BA, BSA and FLA respectively.

●——● BA

■——■ FLA

▽----▽ BSA

Fig. 5.4
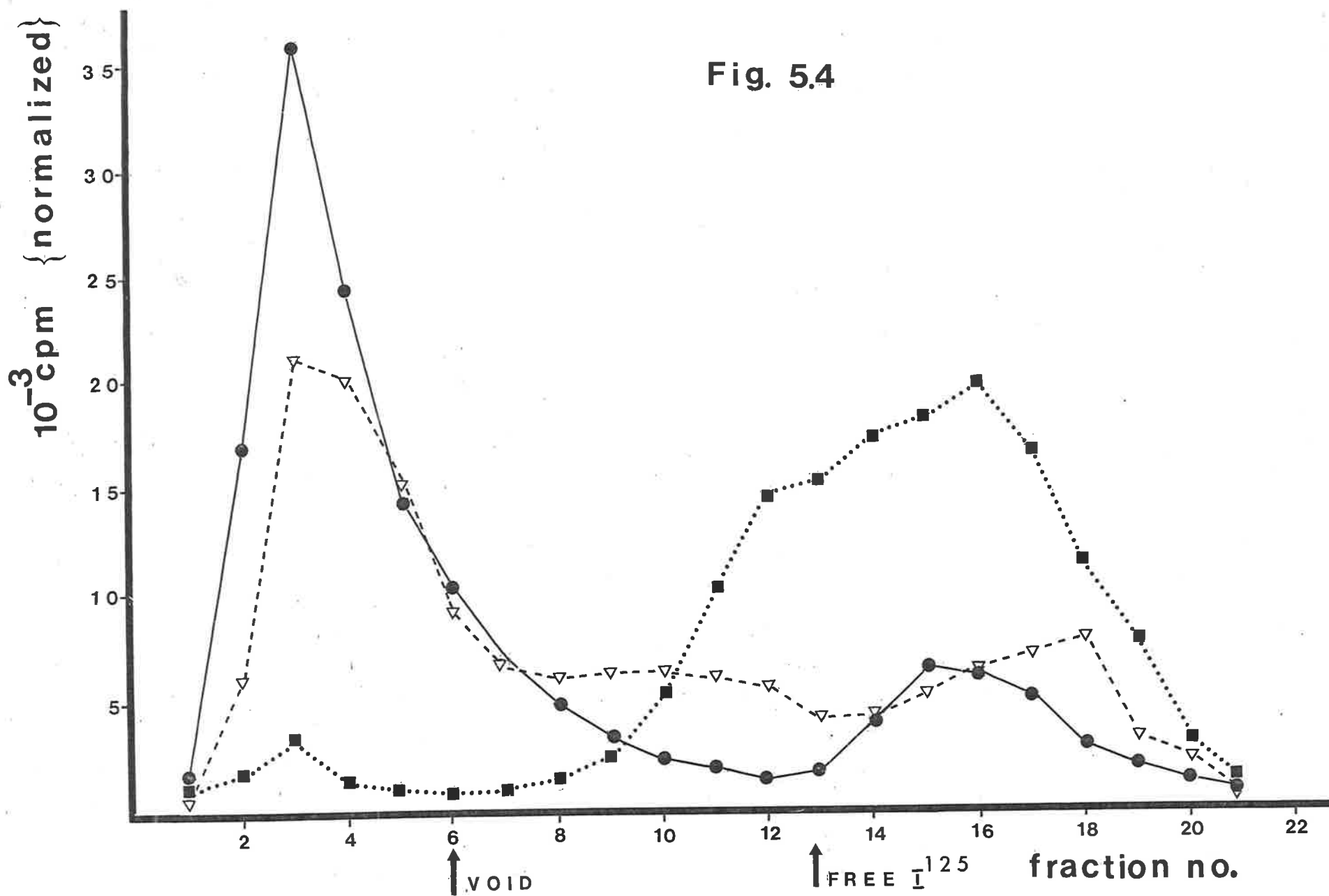
characterized for use in oral absorption studies. The preparation was rather painstaking as the yield was generally low. The two antigens behaved quite differently from each other in both in vivo and in vitro studies.

Parish and Ada (1972) mentioned extensive breakdown of S.adelaide flagellin on prolonged peptic and tryptic digestion. We also found it to be highly degradable in mouse intestinal juice. BA, on the other hand, was relatively resistant to this action. This probably is due to differences in the chemical nature of these antigens. FLA is >95% protein and <0.02% carbohydrate, while the BA preparation studied (#abc) has a protein : carbohydrate content of 1 : 26 (Table 5.1). (Whether other BA preparations with higher protein content behaved similarly is uncertain.) However, the protein content may not be the only factor governing degradability in intestinal juice since BSA, a pure protein, was markedly less degradable than FLA. Obviously, the tertiary structure and the presence of certain amino-acids in the protein are important considerations. Interestingly, the relative degradabilities of BA and FLA in vitro also parallelled their respective clearance rates in vivo when these were injected intravenously. It may be assumed that the degradation in the liver (presumably by enzymic processes) is similar to that in the intestinal juice for these antigens. It also appears that the ability of the liver to metabolise an antigen is related to its ability to absorb it (viz. higher uptake and higher rate of degradation of FLA compared with BA); this consequently determines the clearance of the antigen from the circulation i.e. the phagocytic index.

The in vivo and in vitro studies discussed above are important and pertinent to the understanding of the oral absorption of these antigens. For instance, it may thus be expected that BA and FLA are absorbed at different rates. The form of absorbed material with FLA may be mostly fragments, and these are mostly absorbed by the liver. In other words, it would be very difficult to detect antigenic FLA in the circulation, particularly at long intervals after antigen administration, and without carbon-blockade. BA, on the other hand, is presumably absorbed with minimal degradation (and hence at a lower rate), and a significant amount of this should circulate in relation to that retained by the liver. (The observations of Thomas and Vaez-Zadeh (1974) should, however, be noted in this respect : the amount of antigen sequestered by the liver absorbed from the oral route may be more than that when given by the femoral vein). Thus, the presumed differences in the kinetics, rates and fates of absorption of these two widely different antigens will hopefully aid us in our general understanding of the oral absorption of macromolecules.

5.6   Conclusions

More FLA was sequestered by the liver than BA when these antigens were administered i.v. to mice. Of the two, FLA was also more readily degraded in this organ and in mouse intestinal juice.

6. CHAPTER 6. THE ORAL ABSORPTION OF MACROMOLECULES FROM THE

ADULT INTESTINE : A COMPARATIVE STUDY OF TECHNIQUES

> "Nobody now doubts that some
> material crosses by this
> (oral) route; it is the amount
> that is in dispute." (Lancet
> 1978)

Though macromolecular absorption from the adult intestine has long been suspected, direct evidence demonstrating it is scarce. As mentioned in 1.3, some studies have been made which were based on detecting absorbed (unlabelled) antigen in the serum of animals by immunoassays (Swarbrick et al 1979; Andre et al 1974; Worthington et al 1974). The amounts detected were generally low. The results obtained with radio-labelled antigens were more variable. While Sanford and Noyes (1958) using Cr^{51} endotoxin in dogs, Parkins et al (1960) using I^{125} HSA in rats, and Le Fevre et al (1977) using I^{125} latexes in mice, could not detect any protein-bound label in the circulation, Thomas and Parrott (1974) and Walker * (1977) found small quantities in their systems. Hemmings and Williams (1978) on the other hand, claimed as much as 40% of their I^{125}-labelled proteins was absorbed as high molecular-weight material in the rat, most of which was found in the tissues. Thus, there are widely differing data on the amount of antigen absorbed. This may reflect the different systems used, as discussed in 1.11. For example, how do in vitro methods compare with in vivo models, in the absorption of the same antigen?

*& Bloch

There are also few studies on the uptake of antigens in tissues, since most studies have been confined to detecting the antigen in serum.

In this chapter, the absorption of BA and FLA (and other antigens) was studied by different techniques. These include both established (popular) approaches, e.g. using radioiodinated antigens and everted gut sac models, and original attempts, e.g. using H^3 DNP-labelled antigens.

6.1 The Absorption of I^{125}-Labelled Antigens in vivo

6.1.1 The absorption of I^{125} antigens in mice

6.1.1.1 The absorption of I^{125} BA in mice

Normal SPF mice were fed 1µg I^{125} BA containing 745,500 cpm. The distribution of radioactivity in a representative animal half-hour after feeding is shown in Table 6.1. This is the total radioactivity recovered in the tissues, blood, etc. More than 90% of the radioactivity in all the internal organs and blood was dialysable (see 6.1.1.2). The dialysable radioactivity in the serum was shown to be free iodine by paper chromatography, using NaI as a marker. The radioactivity in the organs was probably blood-borne; hence, it would be misleading to deduce the amount of antigen absorbed in the tissue based on radioactivity count. However, the radioactivity recovered from the intestinal wash-out, including extracts of the washed intestine, was mainly (>70%) high

Table 6.1   Balance chart for the recovery of radioactivity from mouse fed with I^{125} BA half-hour previously

ITEM	% DOSE*
liver (unperfused)	1.03
mesenteric lymph node	0.09
spleen	0.14
kidneys	0.93
small intestine (washed)	13.07
small intestine (wash-out)	43.47
stomach	6.49
colon + caecum	19.81
blood (0.15 ml)	0.41
carcass (less above)	9.86
cage (wash-off)	4.05
TOTAL	99.35

*   Based on undialysed radioactivity recovered and assuming
745,500 cpm (1 μg) I^{125} BA was fed, determined by
counting mouse in a body counter immediately after
feeding. (However, this amount would be 1.45 x higher
if estimated from a 10 μl sample in a well-type counter.)

molecular weight. Thus, as much as 83% of the antigen could still be recovered from the gastrointestinal tract at this time.

The observations of Owen (1977) and LeFevre et al (1978) that the Peyer's patches are important antigen-sampling sites, were examined. Mice were fed I^{125} BA and sacrificed 2 hours later. The rinsed, small intestine was segmented and counted. The profiles obtained (Fig. 6.1) presumably represented the passage of the bolus, since an even*distribution of radioactivity over the entire intestine was seen at 3 hours post feeding. The greater uptake of radioactivity by the intestine when specific antiserum was given together (B) will be considered in 7.2. There was no convincing evidence from the profiles obtained that the Peyer's patches preferentially take up the antigen.

6.1.1.2   Comparative studies on the absorption of I^{125} antigens

in mice

Three mice per group were fed 910,000 cpm I^{125} BA (7 µg), 770,000 cpm I^{125} FLA (1µg) or 1,660,000 cpm free I^{125}. The absorption of these preparations at different times are shown in Fig. 6.2. (Results pooled for each group). There were marked differences in the rate and kinetics of uptake of these preparations in terms of the total radioactivity recovered in the blood, liver and carcass. More FLA was absorbed than BA, and this is perhaps expected from their in vivo stabilities discussed in Chapter 5. For all preparations, the absorbed radioactivity in both blood and tissues was greatest at 1/2 hour post feeding; this rapidly fell off with time. There was more radioactivity in the

*although low

Fig. 6.1

Mapping of the small intestine on the uptake of I^{125}-BA.

Normal SPF mice were fed I^{125}-BA (780,000 cpm/1 µg) in 20% bicarbonate (A) or 17 µl mouse anti-BA serum (#DIgM) (B). After 2 h, the mice were killed and the small intestines were removed, rinsed and cut into 1 cm segments; the segments were counted. Those segments bearing a Peyer's patch were noted (shaded in the diagram).

The profiles shown are representatives of 6 intestines in each group.

Fig. 6·1
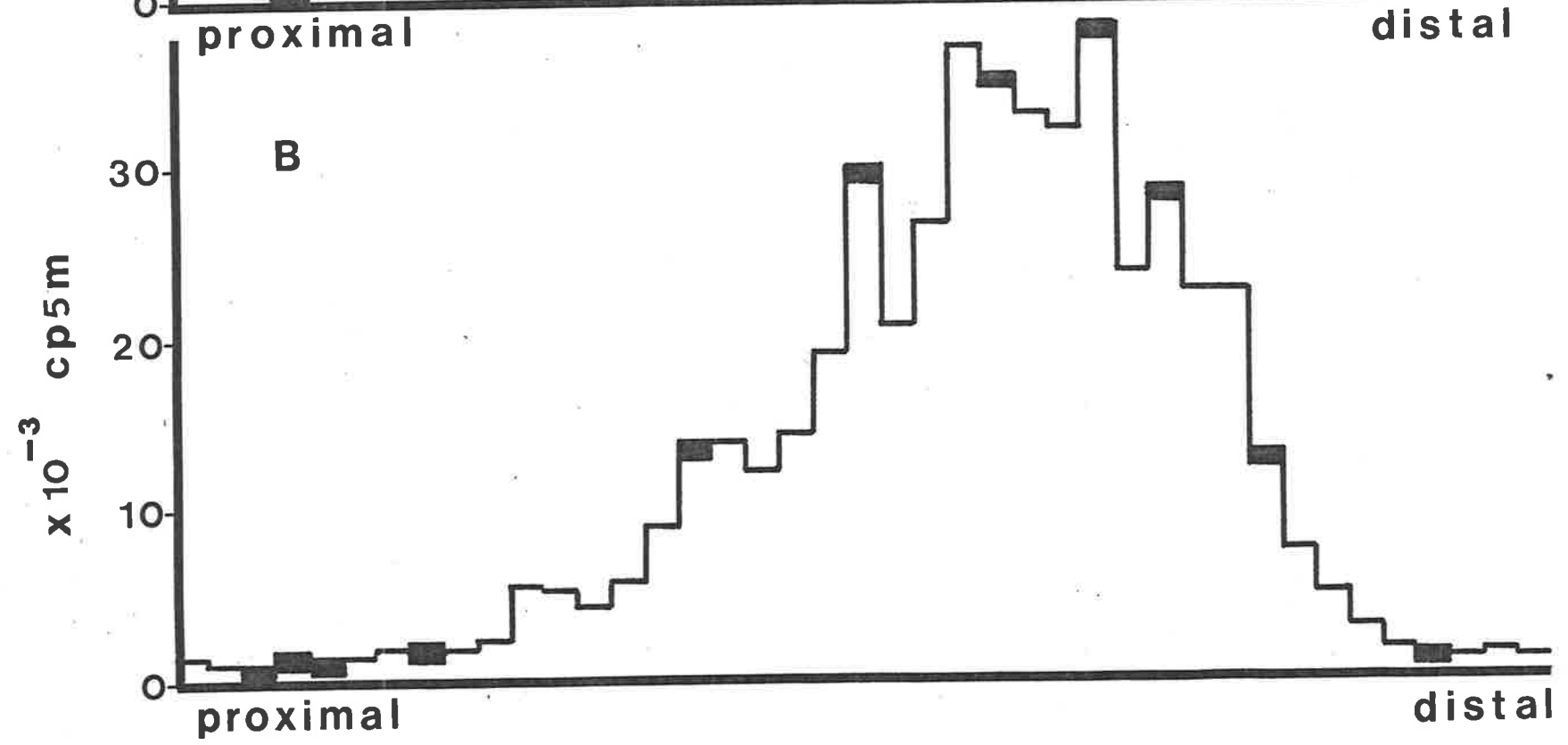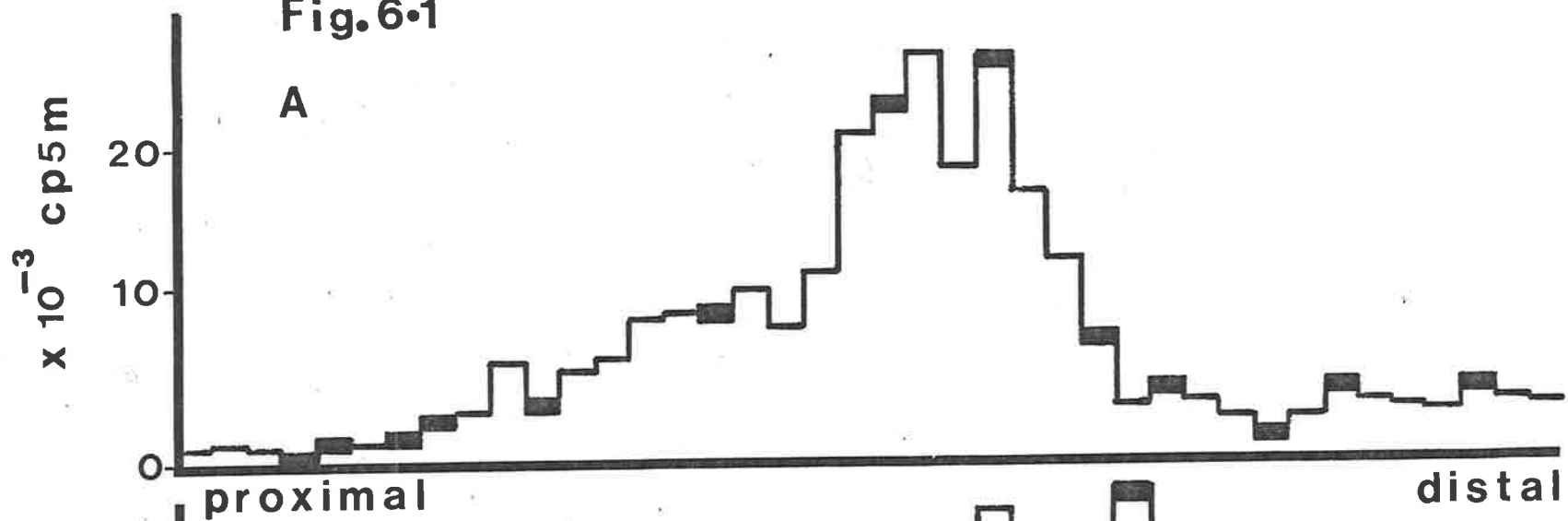
<u>Fig. 6.2</u>

<u>Oral absorption of I^{125}-antigens in adult mice in (A) the perfused liver,</u>
<u>(B) the carcass after removal of the liver, spleen, kidneys and the</u>
<u>gastrointestinal tract (from the stomach to the rectum), and in</u>
<u>(C) 0.1 ml serum.</u>

The numerical values indicate the % non-dialysability of each sample.
(Pool/Average of 3 - 5 mice)
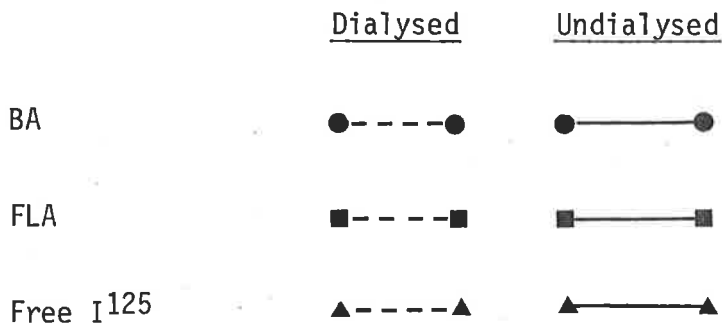
Fig. 6·2
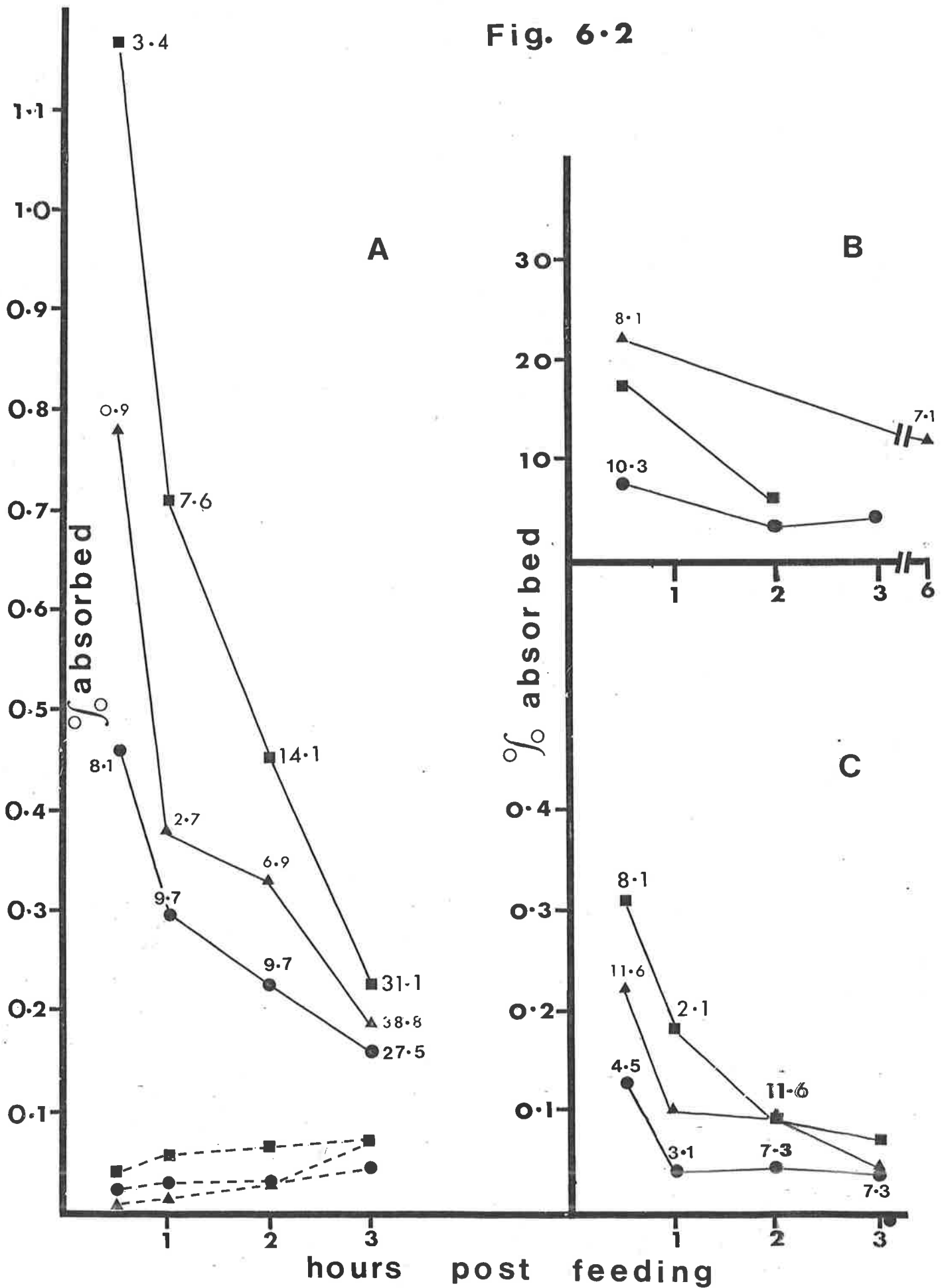
carcass than in the liver or blood; this was especially so in mice given free I^{125}.

However, if the absorption was assessed from the non-dialysable radioactivity in the liver, there was no significant difference in the uptake of the three preparations (Fig. 6.2A). In fact, in all cases, the dialysability of the label in the serum and tissues was very similar. The proportion of non-dialysable radioactivity was generally low (0.9 - 14.1%), but at 3 hours post feeding in the liver, this increased sharply to 31.1% (FLA), 38.8% (free I^{125}) and 27.5% (BA). This suggests reutilization of the label in the organ. In addition, the non-dialysable label recovered from the serum of mice fed with I^{125} BA and I^{125} FLA could not be precipitated by specific antibodies. The efficiency of detecting the original I^{125}-labelled antigen in both these systems was about 70%.

The uncertainty created by using radioiodinated antigens in oral absorption studies was also seen in another study. Mice were fed mixtures of I^{125} BSA and free I^{125} in different proportions. There was no difference in the rates of absorption of these mixtures, as measured by the total radioactivity in the liver (unperfused) and blood (Table 6.2). This is hardly credible, since the absorption rate most certainly depends on the molecular size of the antigen (see 9.1).

6.1.2   The absorption of I^{125} antigens in rats

From the foregoing studies the use of radioiodinated antigens in oral absorption studies in the intact mouse seems questionable.

Table 6.2   Further examination of the validity in using I^{125}-labelled antigens

in oral absorption studies in vivo

ORAL MIXTURE:	GROUPS OF MICE				
I^{125} BSA* (μl)	50.0	25.0	12.5	37.5	0.0
I^{125} free $I^@$ (μl)	0.0	25.0	37.5	12.5	50.0
Total cpm fed x 10^{-5}	4.54	4.51	4.50	4.52	4.49
RECOVERY (% Dose ± S.E.)					
Blood (0.13 ml)	0.34±0.04	0.34±0.01	0.35±0.01	0.33±0.01	0.33±0.03
Liver (unperfused)	0.89±0.07	0.81±0.05	0.81±0.08	0.72±0.05	0.76±0.09

*   9,070 cpm / μl

@   8,970 cpm / μl

Groups of 3-5 normal mice were fed mixtures of I^{125}BSA and free I^{125} in different proportions and then killed 30 min later. The total radioactivity in 0.13 ml blood and whole liver was determined.

However, numerous studies (Hemmings and Williams 1978; Walker et al 1977; Thomas and Parrott 1974) have reported the recovery of absorbed I^{125} labelled antigens in the rat. In particular, Hemmings and Williams (1978) claimed as much as 40% of the radioactivity in tissue homogenates was precipitable by tungstic acid, and 6 - 20% of the label in the supernatant of the homogenate (obtained after high speed centrifugation) was precipitable by specific antibodies. The rates of absorption for different antigens (rat IgG, the gliadins etc.) in their system were also similar. We therefore investigated to see if the rat was different from the mouse in these studies.

I^{125} IgG (MOPC 21) was administered intragastrically to anaesthetized rats. After 1 or 6 hours, the serum and the body tissues were obtained. The amount of total radioactivity, non-dialysable radioactivity and the precipitability [*] by trichloroacetic acid (TCA) in these samples were determined. The results obtained are shown in Table 6.3.

In two respects, the findings in the rat are similar to those of Hemmings and Williams (1978). Firstly, the bulk of the radioactivity was found in tissues, namely, in the carcass. Secondly, the precipitability of the label in all tissue homogenates (liver, carcass, spleen, kidney) by 10% TCA appeared high (11.4 - 42.6%). However, if the precipitate was washed with 10% TCA or PBS, the associated radioactivity could be successively reduced. The residual amount obtained after 2 washes (after an 8-fold reduction in the case of the 6 h-carcass) approximated the non-dialysable radioactivity in the extract (0.0 - 16.0%, of total radioactivity in extract). The marked non-specific precipitation

[*] *of the label*

Table 6.3   Oral Absorption of I^{125} Antigens in Adult Rats

No. animals:	2	2	1
Time of Kill (h):	1	6	6
Antigen fed:	mouse IgG	mouse IgG	BA
Dose given: $X10^{-7}$ cpm	8.1	8.1	2.1
: mg	0.031	0.031	4.0
Recovery of Label in ($\pm$S.E):			

SERUM

1.Total nd[**] (x 10^3)	2.06±1.41	13.92±3.34	1.78
2.quotient[+] (x 10^5)	2.05±1.36	13.70±3.36	2.10
3.% nd[#]	0.95±0.35	3.50±0.30	1.70

LIVER

1.Total[*]	0.27±0.11	0.50±0.05	0.18
2.% nd[#]	0.73±0.03	14.80	16.00
3.% ppt[@] (unwashed)	32.60±0.29	37.67±0.47	42.60
4.% ppt[@] (washed once)	ND	23.07±0.28	ND
5.% ppt[@] (washed twice)	ND	18.54±0.06	ND

CARCASS

1.Total[*]	1.85	7.40	3.00
2.% nd[#]	0.00	0.53	0.00
3.% ppt[@] (unwashed)	21.10	17.12±0.88	11.40
4.% ppt[@] (washed once)	ND	4.24±0.16	ND
5.% ppt[@] (washed twice)	ND	2.53±0.29	ND

SPLEEN

1.Total[*]	0.02±0.01	0.06±0.02	0.04
2.% nd[#]	1.85±0.05	5.40±3.54	2.76
3.% ppt[@] (unwashed)	20.05±0.35	19.00±2.73	13.97

KIDNEY

1.Total[*]	0.20±0.07	0.35±0.03	0.07
2.% nd[*]	1.05±0.15	3.15±0.46	3.20
3.% ppt[@] (unwashed)	26.85±0.35	29.10±2.12	25.90

ND = not done

[**] Total nd radioactivity recovered in 1 ml serum (% dose)

[*] Total radioactivity recovered (% dose, undialysed)

[+] Serum nd cpm ml^{-1} / dose fed cpm ml^{-1}

[#] Non-dialysable (radioactivity)

[@] Precipitability of radioactivity in extract by 10% TCA, with or without successive washing of the precipitate.

Table 6.4   Further studies on the 6 h – liver and muscle

extracts of rats fed with I^{125} IgG

	PELLET (% extract)	SUPERNATANT (% ppty)	
		(unwashed)	(washed once)
LIVER	45.0	22.57	8.39
MUSCLE	15.6	9.12	1.26

The % radioactivity present in the pellet after
centrifuging the extract at 100,000g / 30 min
was determined. The % precipitability (ppty) of the
radioactivity in the supernatant by 10% TCA was also
determined, with or without washing of the ppt.

(See Table 6.3).

Fig. 6.3

Fractionation on Sephadex G200 of (a) I^{125}-mouse IgG (MOPC 21) and (b)the non-dialysable label recovered from the serum of rats 6 h   after being fed this antigen.

□————□   original antigen

■·········■   recovered label from serum (after dialysis)

Fig. 6·3
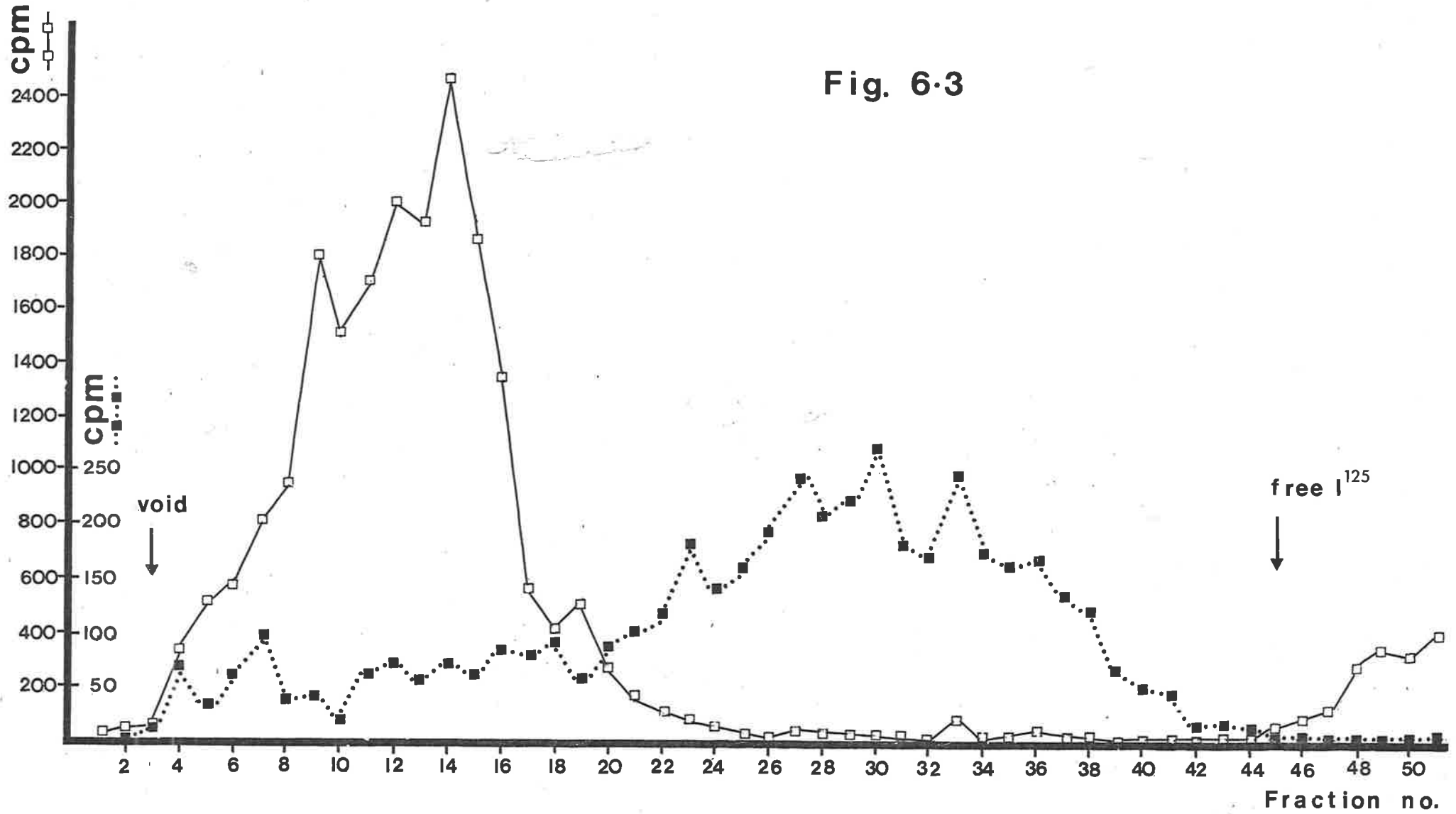
was also seen with the supernatant obtained from high speed centrifugation of the liver and carcass homogenate (Table 6.4). Furthermore, the non-dialysable label obtained in the 6 h-serum and tissue supernatant (containing about 3,000 cpm) was not precipitated by specific antibodies in a Farr assay. In this system, purified goat anti-mouse IgLC was used, which could precipitate $64.9 \pm 4.8\%$ of the original I^{125} IgG and detect less than 0.3 ng (700 cpm) of the antigen. The I^{125} associated-material in the serum was also characterized on Sephadex G200. As seen in Fig. 6.3, it was heterogenous, and there was no indication of the presence of the original antigen.

The absorption of I^{125} BA was also studied in the rat. The results obtained (Table 6.3) were similar to those using I^{125} IgG, except that the absorption here (in terms of total radioactivity) was considerably lower. As with I^{125} IgG, the non-dialysable radioactivity obtained in the serum was not precipitated by specific antibodies in a Farr assay system able to precipitate 75.7% of the original I^{125} BA.

6.2   The Absorption of H^3 DNP-antigens in the Intact Mouse

The absorption of H^3 DNP-labelled compounds (BA, FLA and lysine) was studied in the intact mouse. The results obtained are shown in Figs. 6.4 and 6.5.

There were important differences between the absorption of H^3 DNP-BA and H^3 DNP-FLA, based on the non-dialysable radioactivity. At 1/2 h post feeding, there was a significantly higher uptake of FLA than BA in the liver (55-fold difference) and in the serum

Fig. 6.4

Oral absorption of H³-DNP-labelled compounds in adult mice *in vivo*.

Dose fed/mouse: 333,000 cpm (5 mg) H³-DNP-BA   (A)
                 575,000 cpm (0.8 mg) H³-DNP-FLA   (B)
                 658,000 cpm (40 mg) H³-DNP-lysine (C)

The recovery of H³ label in the different sites at various times was
determined; 0.5 ml blood and the individual organs were pooled from 6
mice in (A) and 3 mice in (B & C). The tissues were extracted as described
in 4.7.7 and 4.7.9. The non-dialysable radioactivity from 0.1 ml serum
or 1 mg dry tissue is expressed as a % of the dose administered. The
numerical values appended to the serum curves indicate the % non-
dialysability of the radioactivity present, while those appended to the
curves in Fig. 6.4 A(b) indicate the % recovery in the supernatants, of
the total non-dialysable radioactivity in the intestinal extracts.

●┄┄┄┄● serum (0.1 ml)   ▽━━━━▽ node (30 mg)@

▲━━━━▲ liver (400 mg)@   O━━━━O Peyer's patches (5 mg)@

□┄┄┄┄□ kidney (120 mg)@   ■━━━━■ non-pp tissue

O╶╶╶╶O spleen (30 mg)@

                              @ average dry weight of organ/mouse

Fig. 6·4
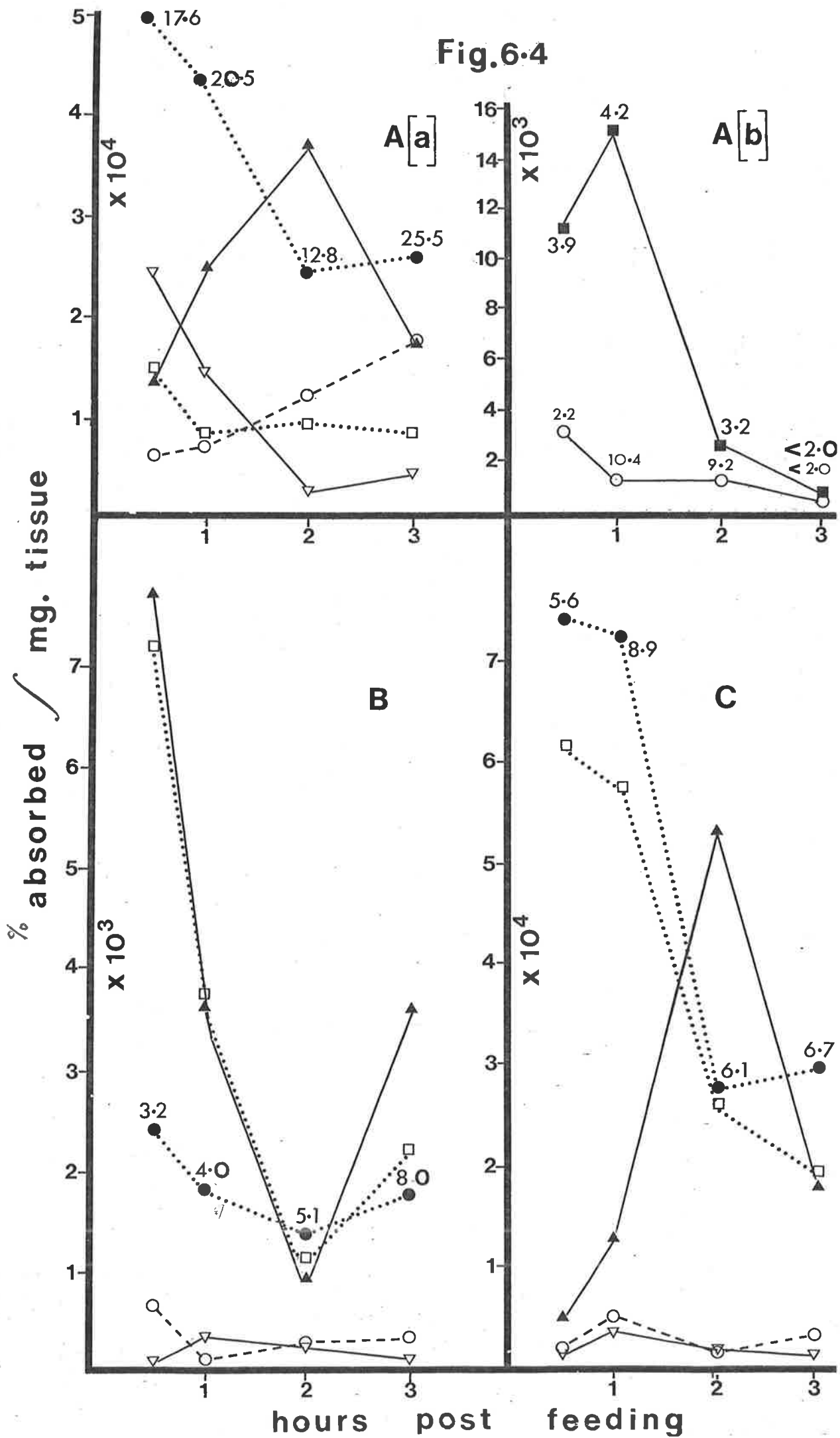
Fig. 6.5

The uptake of H^3-DNP-labelled compounds by whole livers of mice following oral feeding.

The results obtained in Fig. 6.3 for the liver uptake are expressed here as total organ absorption. The numerical values indicate the % non-dialysability of the label in the tissue.

	Dialysed	Undialysed
H^3-DNP-FLA	■- - - -■	■————■
H^3-DNP-LYSINE	▲- - - -▲	▲————▲
H^3-DNP-BA	●- - - -●	●————●
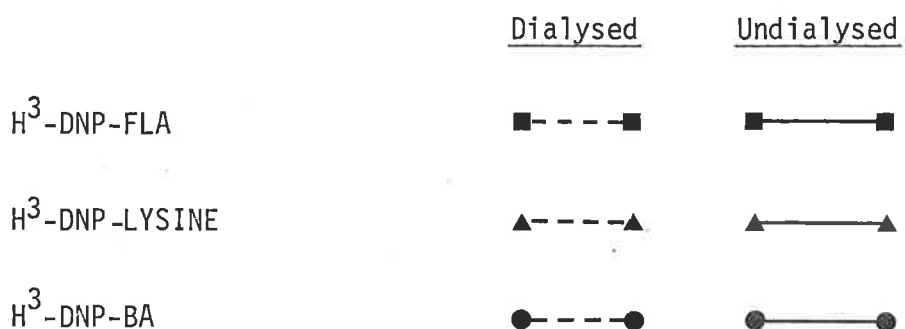
Fig. 6.5
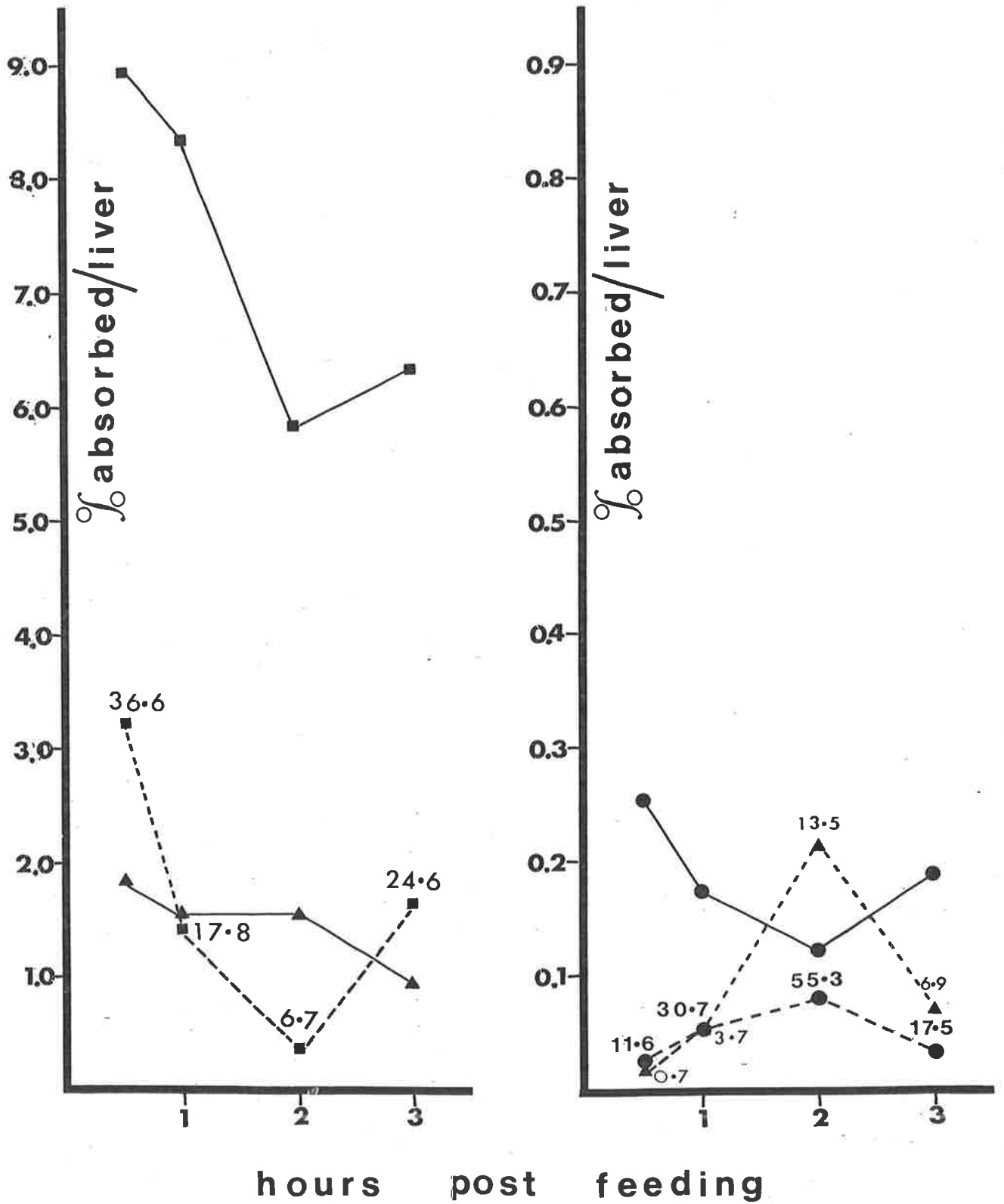
(5-fold). At the same time, the liver appeared more effective in sequestering FLA than BA. This is denoted by the ratio, liver (total) cpm : serum (0.1 ml) cpm, which was 1375 : 1 for FLA and 60 : 1 for BA. Further, the kinetics of uptake of the two antigens by the liver were different. The absorption of FLA (Fig. 6.4B) was maximal early at 1/2 h, and declined rapidly with time. The proportion of radioactivity that was non-dialysable in the liver homogenate also decreased from 36.6% at 1/2 h to 6.7% at 2 h (Fig. 6.5). In contrast, the absorption of BA (Fig. 6.4Aa), increased from 0.00013% at 1/2 h to 0.00037% at 2 h. This probably indicates a slower absorption of the antigen from the gut. It also suggests that the absorbed antigen is perhaps not readily degraded in the liver. In fact, the % non-dialysability of the radioactivity here increased from 11.6% at 1/2 h to 55.3% at 2 h.

With both BA and FLA, the amount of non-dialysable radioactivity in the serum was maximal at 1/2 h. However, the % non-dialysable serum radioactivity was higher for BA (12.8 - 25.5%) than FLA (3.2 - 8.0%) at all times. Further, whilst 7.8 - 20.0% of the non-dialysable BA was precipitable by specific antibodies in a Farr assay, none of FLA was precipitated. (The precipitabilities of the original H^3DNP - antigens were 65.1 ± 0.3% and 54.9 ± 3.8% respectively).

The mesenteric lymph nodes seemed important in the early absorption of BA while in contrast, the spleen showed increasing absorption with time. There was little uptake in the kidneys, unlike the high absorptive capacity of this organ for FLA.

In order to assess whether the non-dialysable radioactivity

obtained with H^3DNP-BA and -FLA truly represents the original antigen, the absorption of a low molecular-weight compound, H^3 DNP-lysine, was studied. (The preparation was made by reacting 75 μg H^3DNFB with 120 mg (excess) lysine, and any unreacted H^3DNFB remaining was not removed). As shown in Fig 6.4C, there was indeed a small amount of non-dialysable radioactivity in the liver, kidney and serum, which presumably was due to reutilization or incorporation of the label by host tissues. However, this amount was relatively small (e.g. note the liver uptake at 1/2 h) and the % non-dialysability of the label in both the liver and serum was low (0.7 - 13.5%) at all times. Thus, it seems reasonable to believe that the non-dialysable radioactivity obtained with BA and FLA denotes true absorption of macromolecules.

The absorption of H^3 DNP-BA by the small intestine was also observed in the same group of mice used in Fig. 6.4Aa. As shown in Fig. 6.4Ab, it is surprising that the Peyer's patches contained less non-dialysable radioactivity than an equivalent mass of intestinal tissue not containing a Peyer's patch, at all times studied. Very little radioactivity was recovered from the whole intestine at 3 h.

6.3   The Absorption of Unlabelled Antigens in the Intact Mouse

6.3.1   Of BA using I^{125} specific antibody injected i.v.

The approach that is used here to detect the absorption of cold, unlabelled BA in the whole mouse, was discussed at length in

3.3.1.

Normal SPF mice were fed 4x0.5 ml BA (36 mg total) or saline in a schedule outlined in Fig. 6.7. Half-hour after the last dose, I^{125} anti-BA antibody was injected i.v.. 15 min later, the mice were bled and killed. The perfused liver and other organs were counted. The index, LP (liver cpm/plasma cpm), was used to gauge the absorption of BA. It is apparent from Table 6.5 (Exp. #3A), that mice fed the antigen had an index (LP=3.87 ± 0.15) that was significantly different (P=0.0085) from that of a control group, given saline only (LP=3.07 ± 0.11). The amount of antigen absorbed was estimated (and corrected) from a standard curve (Fig. 6.6A), constructed from the same batch of mice injected i.v. with graded doses of BA mixed with the I^{125} antibody. Thus, the amount absorbed was 31.1 µg, or 0.08(6)% of the amount given. In a repeat experiment (#3B), the absorption of BA was again demonstrated (LP=3.98 ± 0.17 and 3.30 ± 0.09 for the experimental and control groups respectively; P= 0.0074). The % absorbed was 0.09(0).

The experiment was repeated in germ-free mice to see if the absorption was different from that of SPF mice. The results in Table 6.5 (Exp. #4A) show that 0.07(7)% of the BA fed was absorbed in these mice (LP=3.70 ± 0.11 and 3.11 ± 0.07 for the experimental and control groups respectively; P= 0.004), estimated from a standard curve (Fig. 6.6B) using these mice.

6.3.2   Absorption studies using unlabelled antigen and the

inhibition of haemagglutination

Table 6.5 Detection of intestinally absorbed (cold) BA using I^{125} anti-BA antibody[#] injected i.v.

Exp	Type /No. Mice	BA[*] fed (mg)	DISTRIBUTION OF RADIOACTIVITY (% dose ± S.E.)					LP index[@]	P (LP)
			Plasma	RBC	Liver	Spleen	Kidney		
3A	SPF/5	36	2.73±0.11	0.96±0.05	10.83±0.44	0.55±0.04	2.08±0.08	3.87±0.15	0.008
	SPF/5	0	3.13±0.09	0.99±0.09	9.56±0.20	0.61±0.04	2.09±0.07	3.07±0.11	
3B	SPF/6	45	2.81±0.07	0.81±0.02	11.13±0.27	0.57±0.03	2.24±0.8	3.98±0.17	0.007
	SPF/7	0	3.15±0.07	0.85±0.03	10.38±0.11	0.55±0.01	2.07±0.03	3.30±0.09	
4A	GF/5	31	2.91±0.09	3.65±0.26	10.74±0.28	0.80±0.04	2.55±0.18	3.70±0.11	0.004
	GF/5	0	3.34±0.07	1.57±0.12	10.36±0.15	0.78±0.02	2.31±0.14	3.11±0.07	
4B	GF/4	31+IIJ	3.56±0.40	1.89±0.25	11.38±0.09	0.91±0.07	2.53±0.19	3.24±0.19	0.029
	GF/4	31+NIJ	2.43±0.22	2.00±0.21	10.49±0.96	0.82±0.07	2.58±0.11	4.33±0.26	

[#] ca. 32,000 cpm (0.8µg) in Exp.3 and 65,000 cpm (1µg) in Exp.4; injected half-hour after last oral dose and followed for 15 min. See Fig. 6.7.

[*] Fed half-hourly, with 5-6 doses of BA in saline, NIJ or IIJ, or saline only. (BA#578 & #678 in Exp.3 & 4 resp.)

[@] Measure of antigen uptake: Liver (perfused) cpm / plasma (from 0.13 ml blood) cpm.

Fig. 6.6

Standard curve for quantitating BA *in vivo* in specific pathogen-free
mice (A) and in germ-free mice (B).

I^{125}-mouse anti-BA antibody* was mixed with 0 - 200 µg BA and immediately
injected *i.v.* into normal mice. After 15 min , 0.13 ml blood was obtained
and diluted into 0.15 ml Hanks medium containing 10 U/ml heparin.
0.175 ml of the diluted plasma was counted. The liver was perfused, excised
and counted. The index, *liver cpm/plasma cpm*, was plotted against the dose
of BA injected.

* *ca.* 32,000 cpm (0.4 µg) in (A)
      65,000 cpm (1 µg) in (B)

Fig 6·6
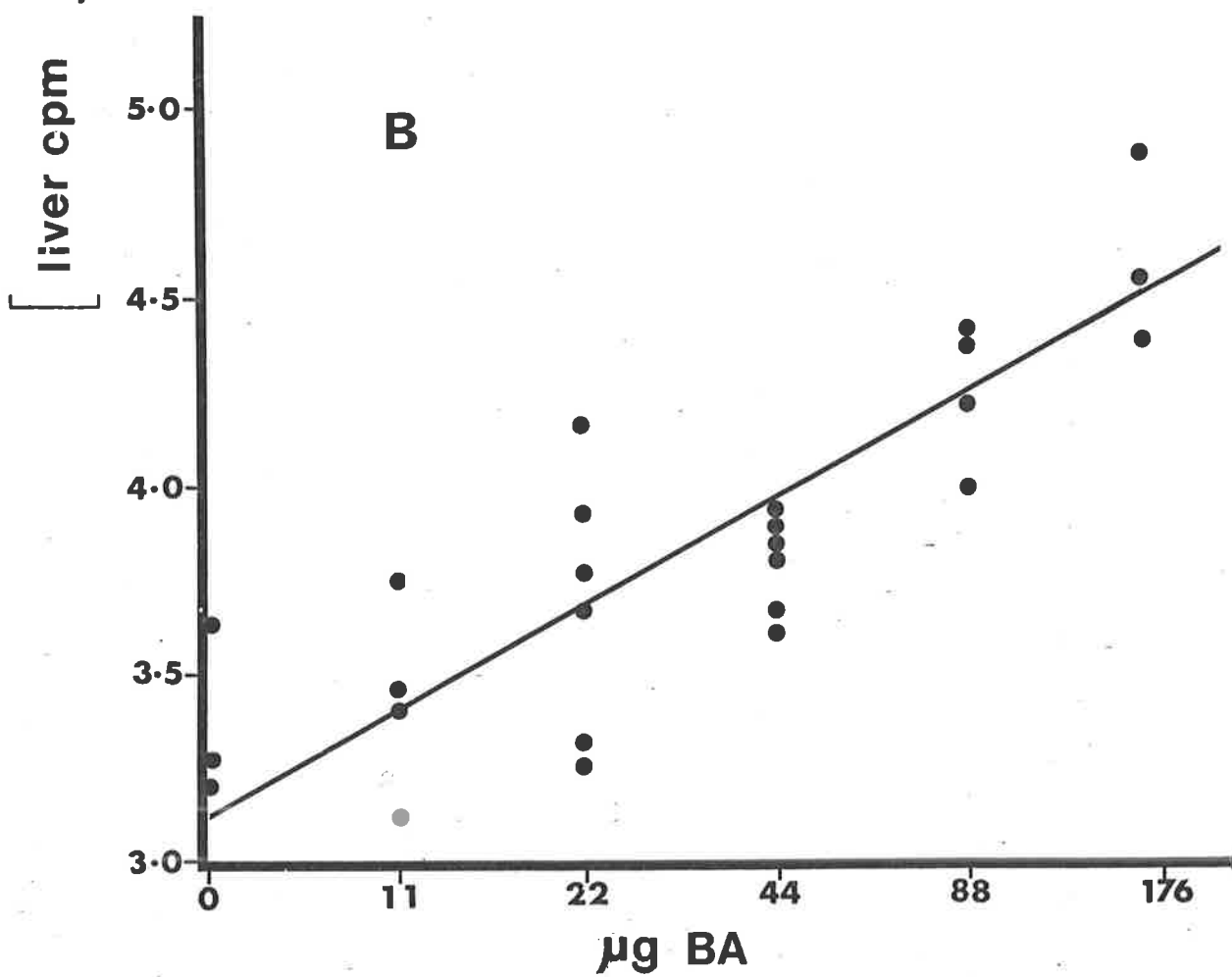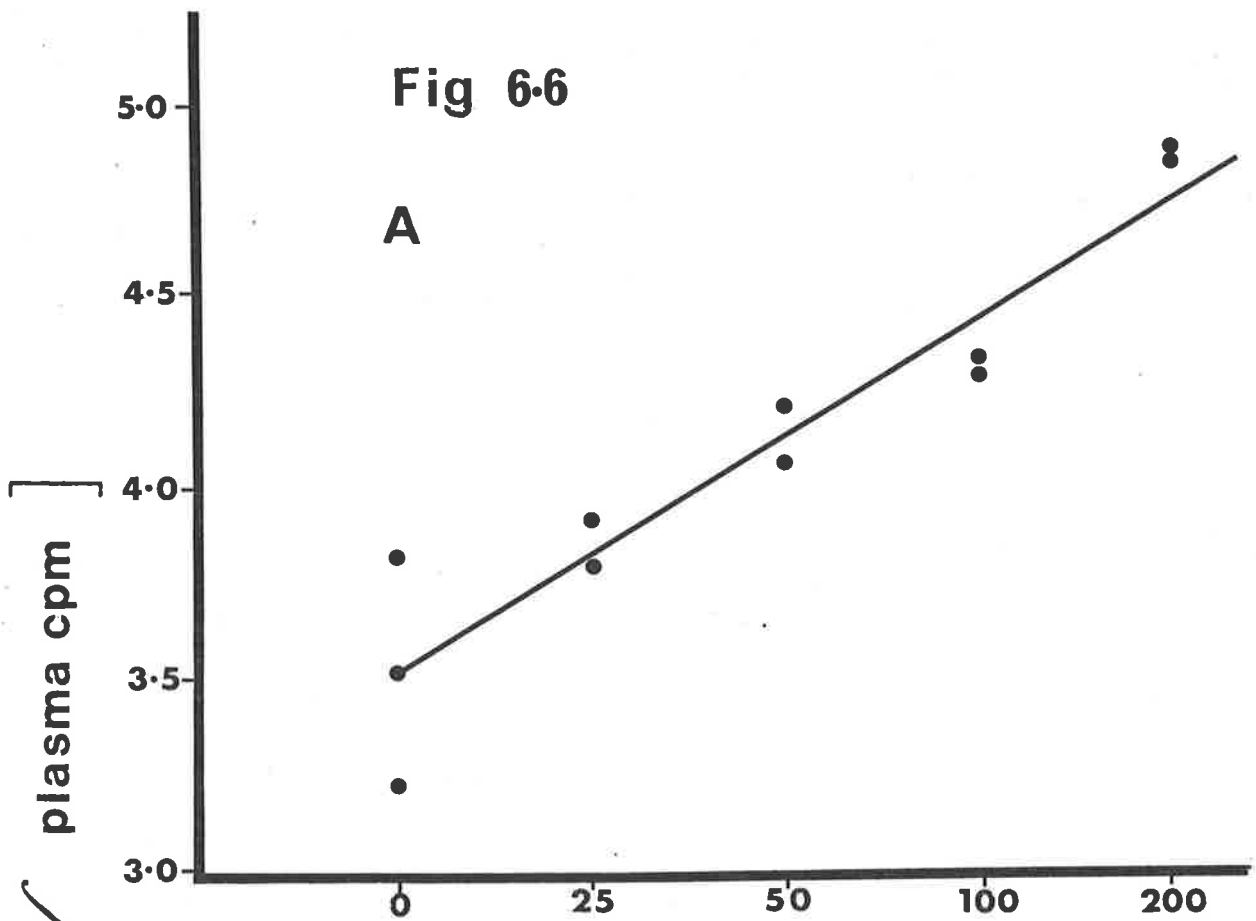
Fig. 6.7

Protocol used in studying the absorption of cold BA using I^{125}-specific antibody in vivo.

I^{125}-antibody
injected i.v.
(0.2 ml)

antibody fed*

time (min)   (-5)__(0)_____(30)_____(55)__(60)_____(120)_____(150)__(165)

antigen fed@   1st   2nd   3rd   4th   animal
                                                             sacrificed
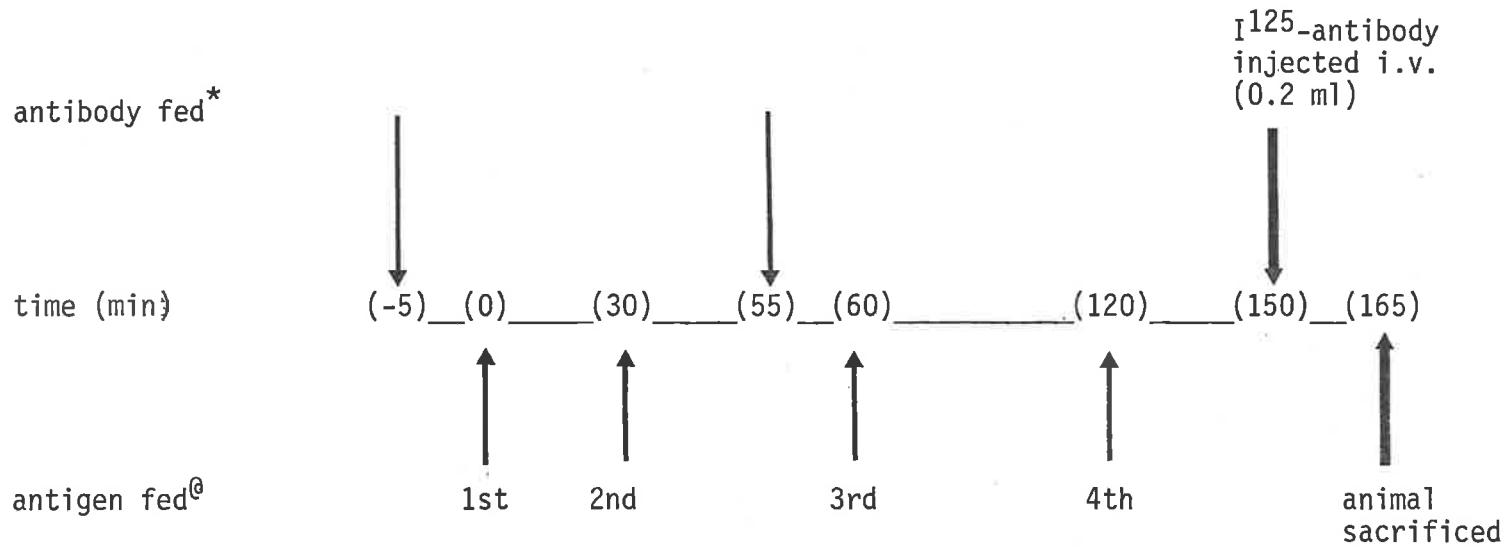
*   0.2 ml normal mouse intestinal juice, immune intestianl juice or mouse antiserum (Expt. 4B only).
@   0.5 ml antigen-20% bicarbonate or (in Expt. 4B) antigen-antibody mixture.

The inhibition of passive haemagglutination (3.1.1) was used to detect absorbed antigens in the early absorption studies. However, no activity was detected in the serum of carbon-blockaded mice following the feedings of BSA (0.5 mg), S.adelaide LPS (8 mg) and FLA (flagella) (1 mg) up to 45 min post feeding. The sensitivities of the technique for these antigens were 625, 190 and 90 ng/ml respectively i.e. less than 0.5, 0.02 and 0.07% of the respective antigens were absorbed/ml serum. Further, less than 2.3% FLA was absorbed into the thoracic lymph of a mouse between 0-13 h, following the oral administration of 1 mg antigen. (The poor sensitivity here is due to the need to use lymph at a dilution greater than 1:256 in the assay).

6.4 The Absorption of Antigens in Everted Gut Sacs

An in vitro model using everted gut sacs was also used to study the absorption of antigens. The results obtained using I^{125} antigens were similar to the in vivo findings in two respects:
(1) The % non-dialysable radioactivity obtained in both the gut sacs and serum was similar and low. From everted gut sac studies, the degraded material was more evident in the jejunum than in the ileum (Table 6.6).
(2) Absorption (in terms of total and non-dialysable radioactivity) was different for different I^{125} antigens in the sac model. Thus, BSA was absorbed 2-4 times more than BA (Table 6.6). Similarly, the total radioactivity recovered in the serum of mice fed with I^{125} BSA 1/2 h previously, was about 3.5 times higher than that obtained wth I^{125} BA (6.1.1.2).

Table 6.6   The absorption of I^{125}-labelled antigens in

everted gut sacs

| I^{125} ANTIGEN IN BATH | | | S A C | | % A B S O R B E D [*] | |
type	cpm/ml	µg/ml	type	no.	jejunum	ileum
BA[#]	275000	0.6	normal	3	0.93±0.16	1.11±0.19
BA[#]	275000	0.6	immune	3	0.74±0.04	0.82±0.12
BA	275000	1600	normal	3	0.39±0.04	0.47±0.07
BSA[@]	286000	0.6	normal	4	1.89±0.34	3.90±0.82
BSA[+]	286000	0.6	normal	4	2.08±0.11	4.19±0.62
BSA[x]	277000	0.6	normal	6	ND	17.99±0.98

[*]   Non-dialysable radioactivity in sac contents obtained
from sacs that were incubated in the bath for 1 h /
37°C. Expressed as: (cpm recovered ÷ cpm in 0.5 ml bath) %
± S.E.

[#]   Bath also contained 1mg/ml BSA

[@]   Bath also contained 1.6mg/ml BA

[+]   Bath also contained 1mg/ml KLH (haemocyanin)

[x]   Bath also contained 6mg/ml LPS; taken from Fig. 7.2 for
control sacs, which were pre-incubated in Hanks medium
for 18 h / 4°C.

However, the gut sac model differs from the in vivo situation in one important aspect. While the radioactivity in the serum of mice fed with I^{125} BA was not precipitable by specific antibodies, 10-76% of the dialysed label obtained from sac contents using I^{125} BA was precipitated by specific antibodies in a Farr assay, that could precipitate 75.7% of the native antigen (Table 7.2). The absorbed material in the sac using H^3 DNP-BA was similarly precipitable.

The amounts of both BA and BSA were higher in the ileum than in the jejunum (Tables 6.6, 7.2). This may be due to the lesser amount of mucus observed in the former (judged visually). Indeed, removal of the mucus with 1mM dithiothreitol (Sigma) (Table 7.2) or by prolonged pre-incubation of the intestine in Hanks medium (Table 6.6) increased the absorption considerably.

6.5   The Functional Demonstration of the Oral Absorption of

Antigens

There are numerous ways in which the intestinal absorption of macromolecules can be demonstrated functionally in vivo. For instance, Danforth and Moore (1959) observed the concommitant depression of blood glucose in rats fed with insulin. Also, Berstein and Ovary (1968) induced passive cutaneous anaphylaxis in guinea-pigs with oral feedings of DNP-lysine and egg-albumin.

Our own approach was based on the induction of fatal systemic anaphylaxis in mice to BSA. Mice were immunized against BSA using the following schedule. 0.4 mg BSA was injected intraperitoneally

with 0.2 mg Evans Blue as adjuvant (Arora and Crowle 1978). Two booster doses of 0.4 mg BSA (each) in saline were given i.p., 4 and 7 days following the primer. The mice were used in experiments 1 week after the last booster. At this stage, they showed a heightened reactivity to the antigen. With as little as 1 µg injected i.v., the mice showed signs of anaphylaxis, manifested by restlessness, laboured respiration, etc. A change in the intestine was shown by the greater leakage of i.v.-injected I^{125} keyhole limpet haemocyanin (KLH) into the gut (see 3.3.2 and 7.3). The mice died instantaneously when injected with 0.2 mg BSA i.v., though 0.1 mg was tolerated. The question we sought to answer was : would the mice die if challenged with the antigen orally? Indeed, all 4 mice died when fed with massive doses of BSA, given as 4 x 0.5 ml half-hourly doses of 10% BSA in 20% bicarbonate. (Another group of mice survived when given 3 x 0.5 ml half-hourly doses of the mixture, i.e. 150 mg BSA total). The lungs of these mice were expanded and the amount of I^{125} KLH (100,000 cpm) recovered in the intestine was $0.47 \pm 0.05\%$ of the dose administered. Four normal mice, given the same treatment, remained healthy throughout the study, and the gut permeability was normal $(0.21 \pm 0.02\%)(P=0.0125)$.

6.6   Discussion

There is little doubt from the various models described here that macromolecules are absorbed from the adult mouse intestine. In general, the different models are comparable with regards to the rates of absorption of different antigens. For instance, more FLA was absorbed than BA in the intact mouse, whether the antigens were labelled with I^{125} (in terms of total radioactivity

absorbed, see below) or with H^3 DNP (in terms of total or non-dialysable radioactivity). Further, more I^{125} BSA was absorbed than I^{125} BA in both the intact animal (total radioactivity) and in everted gut sacs (total or non-dialysable radioactivity).

It is difficult to make meaningful comparisons of antigen absorption in the different systems, since the conditions and methods of assay are rather different. However, it is safe to say that the absorption of intact BA, as observed in all these models, is very small. It is not valid to assess this rate using I^{125} BA _in vivo_ (see below), though this may be measured in everted gut sacs using the same labelled antigen. In the latter, the amount of macromolecular BA absorbed by the 11 cm-long ileum in 1 h was about 1% (see Tables 6.6 and 7.2), of which 60% was antigenic. The amount of non-dialysable H^3 DNP-BA absorbed in the intact mouse at 1 h was 0.05% of dose fed in the whole liver and 0.004% of the dose fed in 1.0 ml serum, only 8 - 20% of the latter was antigenic. The amount of antigenic BA absorbed in the same type of mice, as measured by I^{125} anti-BA antibody _in vivo_, after 4-5 half-hourly feedings of cold antigen, was 0.09%. Presumably, this method detects the total amount of antigen absorbed from the intestine in the whole animal.

The validity of using I^{125} antigens for measuring oral absorption in the intact mouse and rat is questioned by these studies. Though the total radioactivity absorbed was different between different antigens (e.g. more FLA was absorbed than BA), and probably reflects differences in antigen uptake, no antigen-associated radioactivity could be found with BA, FLA, BSA

(from early studies not cited in the text) or mouse IgG. Further, based on the non-dialysable radioactivity absorbed, there was no difference in the absorption of these antigens or of free I^{125}. These findings taken together, suggest that as soon as the I^{125} antigen is absorbed, it is immediately deiodinated, so that the released label follows the fate of free I^{125} in the blood and tissues (bathed in it). Hence, the major site of deiodination is probably the gut mucosa rather than in the lumen or the internal organs, since the free radioactivity recovered was just as much in the sac contents (Table 7.2) as in thoracic lymph (Table 6.7) or in serum (Fig. 6.2). There is also evidence for the de novo metabolism of the I^{125} label in the animal : (1) Non-dialysable radioactivity was found in both serum and tissues of mice fed with free I^{125}. (2) The % non- dialysability of the radioactivity in the liver of mice fed with I^{125} BA, I^{125} FLA and free I^{125} increased with time, so that the amounts at 3 h post feeding were 27.5, 31.1 and 38.8% respectively. The apparently high precipitability of radioactivity in tissues by tungstic acid as observed by Hemmings and Williams (1978), using I^{125} rat IgG and the gliadins, is probably non-specific. We have similarly observed high precipitability of the I^{125} label in the tissues of rats fed with I^{125} mouse IgG or I^{125} BA by 10% TCA; however, the radioactivity in the precipitate could be successively washed off so that the residual amount approximated that obtained by exhaustive dialysis. This latter technique of separation is useful and valid, as demonstrated in studies where the I^{125} antigen was given i.v. (Fig. 5.3). Unlike Hemmings and Williams (1978), we could not obtain any antigen-associated radioactivity either in the serum or in the supernatant of tissue homogenate after high speed centrifugation.

Table 6.7   Oral absorption of I^{125} BA in the mouse thoracic lymph

TIME LYMPH COLLECTED	VOL (µl) COLLECTED	RADIOACTIVITY RECOVERED		
		cpm/µl	%ABSORBED[*]	%HMW[#]
(-1) to 0[@] h	100	0.0	0.0	0
0 to 1 h	70	4.3	0.036	3
1 to 1.5 h	70	8.1	0.067	3
1.5 to 18 h	300	19.4	0.693	3
Serum at 18 h	20	24.2	0.058	3

[@] Mouse administered orally with 840,000 cpm (1 µg) I^{125} BA at 0 h

[*] Total undialysed radioactivity in sample, % dose

[#] % radioactivity excluded by Sephadex G 25

Although I^{125} antigens are not suitable for oral absorption studies in vivo, they were useful in an in vitro model. Unlike that recovered from the serum, the dialysed radioactivity obtained in everted gut sacs using I^{125} BA, was precipitable by specific antibodies. The reason for this difference is not clear. Perhaps, the deiodinase activity and other local factors affecting absorption e.g. mucus, are diminished in the in vitro set-up. (This may account for the apparently higher absorption of BA in this system compared with those seen in the intact animal.) The differences between the in vitro and in vivo models in the absorption of I^{125} antigens may also be interpreted as a caution in drawing conclusions from everted gut sac studies. In other words, although in vitro models have been shown to be physiological in terms of the viability and function of the intestine, there is no doubt that they do not simulate the in vivo conditions completely.

The unique approach of detecting orally absorbed antigen with a labelled specific antibody in vivo has good potential, despite the great difficulties encountered. Any antigen absorbed from the intestine into the whole animal (including that sequestered by tissues), is presumably detected by this means, which is otherwise difficult or impossible to measure by other techniques. However, this technique requires that optimal amounts of antibody be determined and used, since complexes formed in either antigen or antibody excess are less readily phagocytosed (Thomas and Vaez-Zadeh 1974).

H^3 dinitrophenylation seems a useful tag for oral absorption

studies in vivo, since meaningful results were obtained with H^3 DNP-BA and -FLA. The conjugate appeared stable, and isotopic exchange of the tritium was minimal. The antigenicity of the original compound was also not affected by the conjugation. Though the possibility of the de novo metabolism of free DNP-lysine was indicated, this was minimal and does not invalidate meaningful observations made with H^3DNP - labelled antigens. For example, significant differences were obtained between the absorption of H^3 DNP-BA and H^3 DNP-FLA, which were totally consistent with the in vivo behaviour of these antigens (5.3). Thus, the rate and kinetics of absorption, the nature and fate of the absorbed material, etc. were different, as expected.

It is apparent from the studies with H^3 DNP-antigens that the liver is an important organ in the absorption of gut-derived antigens. This is particularly so for FLA. The kidney also has a similar absorptive (and degradative) capacity for this antigen, but the importance of skeletal muscle has not been determined. However, the latter does not appear to take up much BA, since in a separate study, the absorptive capacity was found to be less than that of the liver (<0.0001% dose/mg tissue at 1 h). The mesenteric lymph nodes have a higher capacity than the liver or other tissues in the absorption of BA at 1/2 h. This implies that the thoracic duct is an important route of absorption for this antigen. Unfortunately, an early attempt to recover this antigen from the lymph was unsuccessful (Table 6.7), since I^{125} BA was used and this was prone to deiodination. The increasing uptake of orally administered BA by the spleen suggests that this organ may be important in the oral induction of immune-responsiveness to this antigen.

The induction of fatal anaphylaxis in hyperimmune mice with large doses of the antigen given orally has important implications.* Though the sites of the lesion were not investigated, they were probably both intestinal (as shown by the greater gut permeability) and systemic (inflated lungs).

6.7   Conclusions

The intestinal absorption of macromolecules, using BA and FLA, was demonstrated in a number of ways. Radioiodination of the antigens was not suitable for the oral studies _in vivo_ while H^3 DNP-labelling proved extremely useful. The absorption of H^3DNP - BA and -FLA was vastly different. Very little BA was absorbed; the absorbed material, mostly of high molecular weight, was sequestered partly by the liver and other tissues, while a relatively large proportion of it appeared in the circulation. In contrast, the absorption of FLA was more marked and rapid; much of the absorbed material ended up in the liver and kidneys, which was rapidly degraded and lost. Consequently, only relatively small amounts of high molecular weight FLA could be recovered from the circulation, none of which was antigenic.

*This is discussed in 9.3 .

7. CHAPTER 7. THE EFFECT OF SPECIFIC ORAL IMMUNIZATION ON THE

ABSORPTION OF MACROMOLECULES

"It has also been stated that,
with repeated feeding of a
certain natural protein, it is
possible to get the opposite
effect, i.e. no absorption.
There may be a local action of
precipitins, which causes
"blockade" in the intestinal
mucosa and acts as a defence."
(Verzar and McDougall 1936)

The possibility that repeated feeding of an antigen may result
in its decreased uptake has been suspected for a long time. A
basis for this was offered by the recent observations of Walker
(1975), Andre et al (1974) and Swarbrick et al (1979), that
specific oral immunization of rats or mice reduced the absorption
of the specific antigen in these animals. Implicated in these
findings is an important function of coproantibodies. More
specifically, this may be a major role of sIgA, whose apparent
biological inertness has so far eluded the teleological reasons
for its predominance in the gastrointestinal secretion of most
mammalian species. The evidences for such a role, however, are
only circumstantial, whereas the more definite involvement of IgG
in the inhibition of antigen uptake has been shown (Walker et al
1972a; Brandtzaeg and Tolo 1977). Consequently, a question that
arises and remains unanswered is why then, should IgA be evolved
and exist predominantly in secretions? Is it more efficient than
other Ig classes in blocking antigen uptake?

This chapter describes observations made with BA and FLA in
normal and orally immunized mice.

7.1   Specific Oral Immunization Inhibits Macromolecular Uptake

The question of whether specific oral immunization affects the absorption of antigens was examined using the different absorption models described in Chapter 6, and an oral immunization schedule outlined in 4.5.7.

7.1.1   Using the I^{125} specific antibody model with immune

intestinal juice passively given to normal germ-free mice

Normal germ-free mice were fed half-hourly with 4 x 0.5 ml BA mixtures. The mixtures contained 31 mg BA (total) and 10% (v/v) of either normal mouse intestinal juice (NIJ) or intestinal juice from mice orally immunized against the antigen (IIJ). 0.2 ml NIJ or IIJ was also fed to the mice 5 min before the first and third antigen doses, as shown in Fig. 6.7. The amount of BA absorbed (expressed as the LP index, 6.3.1) was derived from the distribution of i.v. administered I^{125} anti-BA antibody in the liver and plasma.

It is apparent from Table 6.5 that IIJ inhibited the intestinal absorption of antigenic BA significantly compared with control mixtures using NIJ (P=0.029), to levels below the detection of the system.

This effect was repeated in Table 7.1 using IJ fractionated with 50% saturated ammonium sulphate. Again, IIJ was found to be inhibitory (P=0.040).

Table 7.1 Further studies on the absorption of BA using
I^{125} specific antibody in vivo[#]

MOUSE STRAIN	ORAL CHALLENGE	LIVER CPM / PLASMA CPM (LP ± S.E.)
GF	Saline	3.21 ± 0.31
GF	31 mg BA in NIJ fr[@]	3.96 ± 0.46[*]
GF	31 mg BA in IIJ fr[@]	2.94 ± 0.02[*]
SPF	31 mg BA in NIJ fr[@]	3.78 ± 0.21
SPF	Saline	3.45 ± 0.12

[*] P = 0.0422

[#] As in Table 6.6 using BA # 678, 3 mice / group, and 32,000 cpm I^{125} specific antibody.

[@] IIJ fraction obtained thus: 5 ml IIJ (Farr titre = 1:4) absorbed with 10^{10} fixed S.typhimurium -M206 and precipitated with 50 % sat ammonium sulphate. 0.6 ml (total) pf the ppt suspended in 3 ml PBS was used. NIJ fraction was similarly obtained but absorbed with V.cholerae.

7.1.2 Using everted gut sacs from actively immunized mice

Everted gut sacs were made from the jejunum and ileum of mice that were normal or orally-immunized against BA (IMMUNE). Some of the sacs were pre-treated with 1 mM dithiothreitol ($37^{o}C/15$ min) to remove the mucus before use (see Bull & Brookman 1977). The absorption of I^{125} BA in the various sacs was studied, with the results obtained in Table 7.2 and which may be summarised as follows:-

(1) Based on the non-dialysable radioactivity in the contents of sacs that were not pre-treated with DTT, the absorption of I^{125} BA was generally lower in immune sacs than in normal sacs (see also Table 6.6). This difference was more pronounced in the jejunum in one study (Exp #1; P=0.0238) and in the ileum in another (#2; P=0.0039).

(2) There is a general correlation between the amount of non-dialysable radioactivity recovered in the sac and that in the sac wall (after thorough rinsing). Thus, there was also more radioactivity in the wall of normal sacs than that of the corresponding immune sacs (P=0.0290 for jejunal sacs, Exp #1; P=0.0039 for ileal sacs, Exp #2).

(3) From each intestine, the jejunal sac had less radioactivity than the ileum. On the other hand, there was correspondingly more radioactivity in the rinse of jejunal sacs than that of ileal sacs from both normal and immune intestine; this is probably due to mucus-associated radioactivity, since more mucus (intestinal debris, etc.) settled out in the incubation and rinse tubes containing jejunal sacs.

Table 7.2 Absorption of I^{125}-BA in everted gut sacs[@]

EXP/ mice used	ITEM[*]		UNTREATED SACS normal JEJUNUM	ILEUM	immune JEJUNUM	ILEUM	DTT-TREATED SACS normal JEJUNUM	ILEUM	immune JEJUNUM	ILEUM
I SPF	sac	%rec	0.76±0.09	0.92±0.05	0.32±0.03	0.98±0.09	1.20±0.12	1.67±0.15	0.70±0.13	0.90±0.14
		%nd	4.85	5.40	3.55	6.67	7.72	9.87	7.50	8.25
	rinse	%rec	33.1 ±2.5	18.0 ±1.3	42.5 ±0.9	24.8 ±1.9	34.1 ±4.4	29.8 ±3.0	34.0 ±4.1	24.9 ±1.5
		%nd	45.4	38.2	46.2	43.0	39.2	35.7	50.1	46.4
	wall	%rec	32.1 ±3.6	40.8 ±4.0	19.9 ±2.6	31.6 ±4.4	40.3 ±2.8	52.9 ±4.4	25.4 ±4.6	17.8 ±1.5
IIA SPF	sac	%rec	0.38±0.06	1.01±0.03	0.31±0.06	0.43±0.02	0.60±0.10	0.89±0.06	0.92±0.16	0.98±0.12
		%nd	7.45	14.9	5.91	8.05	13.0	18.3	16.1	22.0
		%ppt	49.6 −0.0	64.6 −4.7	10.4 −0.5	50.2 −4.6	68.8−12.3	72.6−13.3	72.3−24.4	76.3−19.7
	wall	%rec	23.9 ±2.8	30.5 ±2.4	11.1 ±1.5	16.3 ±1.5	28.4 ±1.5	29.5 ±2.0	21.9 ±1.5	22.0 ±1.6
IIB GF	sac	%rec	0.27±0.05	0.84±0.16						
		%nd	5.10	12.5						
		%ppt	74.0−11.5	70.5−13.6						
	wall	%rec	13.3 ±1.1	20.8 ±3.2						

[@] Dithiotreitol (DDT)-treated or untreated gut sacs prepared from the jejunum of ileum of normal or immune mice (4-5 / group; SPF or GF) were incubated in bath containing I^{125}-BA at 37°C for 1 h (see 4.7.8)

[*] sac=sac contents; rinse=pool of rinse solutions; wall=rinsed gut wall; nd=non-dialysable (radioactivity) rec=total radioactivity recovered (nd for sac) ÷ initial radioactivity in 0.5 ml bath.
      (bath conc.=160,000 cpm (533 ng)/ml in Exp.I and 287,000 cpm (290 ng)/ml in Exp.II)
ppt=precipitability of nd label in Farr assay; figures after the{-} sign indicate control precipitation
      without extraneous antibody.   (Other results expressed as mean ± S.E.)

(4) There was no precipitation of the dialysed radioactivity from either normal or immune sacs by 50% saturated ammonium sulphate (without extraneous antibody added). In other words, there were no pre-formed immune-complexes in the contents of these sacs. (See Sections 7.2 and 7.4.)

The effect of pre-treating the intestine with DTT is apparent from Table 7.2. In general, it abolished the difference between the jejunum and ileum in the transport rates of I^{125} BA (in terms of sac content and gut wall radioactivity), and in the rinse for each intestine. It also diminished the difference between the normal and immune intestine in these respects. In other words, the absorption of I^{125} BA in these DTT-pretreated sacs was similar, and approximated to that of the untreated, normal ileum.

7.1.3 Using I^{125} antigens in vivo

The oral absorption of I^{125} BA and I^{125} FLA was measured in both normal and orally immunized mice in vivo. The results are expressed as the total (undialysed) radioactivity recovered in the different tissues, and also as the non-dialysable radioactivity in the plasma (Fig. 7.1, Tables 7.3 and 7.4). Thus, for both antigens, less radioactivity (total) was absorbed in the internal organs and circulation of immune mice than those of normal animals, particularly early after feeding (< 2 h). For instance, at 1/2 h, the total liver count was less in immune than in normal animals for both BA (P=0.029, Fig. 7.1) and FLA (P=0.155, Table 7.4). On the other hand, there was more radioactivity in the washed intestine (see 7.2 below), stomach (except for BA) and colon of immune mice than those of normal mice for both antigens;

Fig. 7.1

The intestinal absorption of I^{125}-BA in normal and immune mice *in vivo*.

Groups of 5 mice that were either normal (– – –) or immune (———)
were fed with 1.08 x 10^6 cpm (1 µg) I^{125}-BA. The recovery of the
undialysed label in the different sites at various times was determined.
The standard errors for the intestine and liver at ½ h are shown, while
the % non-dialysability of the radioactivity in the plasma is also
indicated.

X	small intestine	□	kidney
■	stomach	O	spleen or m. lymph node
△	colon + caecum	●	whole blood (0.13 ml)
▲	liver (unperfused)		

Fig. 7·1
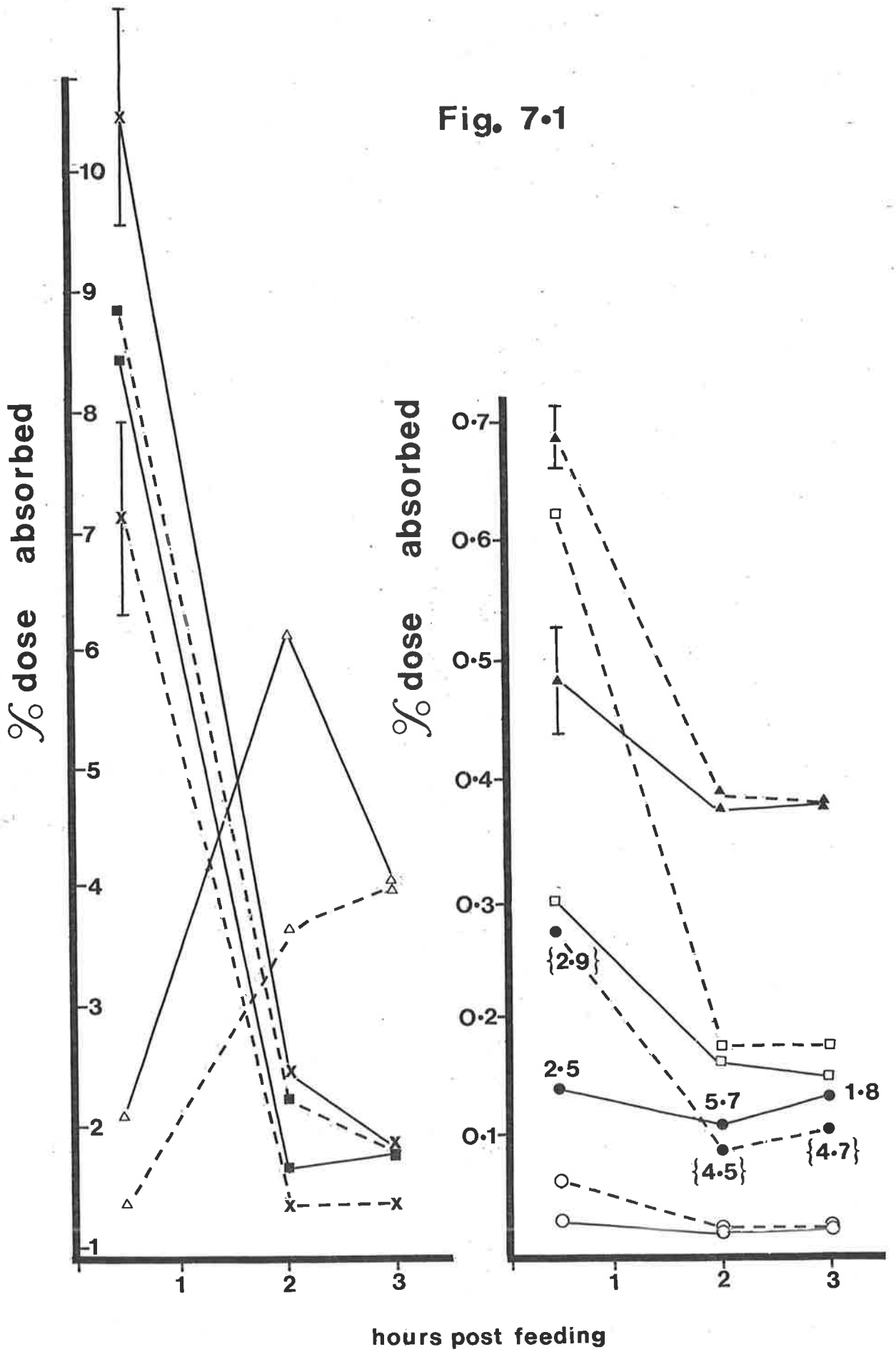
Table 7.3   Further absorption studies using I^{125} BA in the intact mouse

| Exp | BA dose (cpm) $x10^{-6}$ | Time of kill (h) | Status of mice | RECOVERY OF RADIOACTIVITY (%DOSE±S.E.) | | | P(liver) |
				Liver*	Spleen	Plasma@	
EF	1.57	1.5	normal	0.48±0.01	0.032±0.003	0.011	0.0125
			immune	0.38±0.01	0.024±0.003	0.020	
NO	2.04	2.0	normal	0.39±0.02	0.028±0.006	0.021	0.0039
			immune	0.29±0.02	0.018±0.001	0.022	

*   Unperfused, undialysed

@   Obtained from 5 x 0.15 ml blood and dialysed

(5 mice / group)

Table 7.4   Oral absorption of I^{125} FLA in normal and immune mice in vivo

Status /No. mice[#]	Time of kill (min)	RECOVERY OF RADIOACTIVITY (% DOSE FED ± S.E.)[*]									
		Liver	M.Lymph Node	Spleen	Kidney	Intestine	Stomach	Colon	Carcass	Blood	Plasma
NORMAL 5	30	1.65±0.15	0.15	0.12	1.34	4.31±0.32	6.03	1.27	17.1	0.55	0.026 (8.1)
IMMUNE 5	30	1.45±0.06	0.12	0.09	1.28	5.44±0.34	8.80	3.78	15.2	0.47	0.047 (17.6
CONTROL 4	30	1.88	0.15	0.11	1.75	4.54	5.97	1.62	17.9	0.62	0.027 (7.1)
NORMAL 5	120	0.67±0.07	0.05	0.04	0.45	1.45±0.34	2.02	9.93	6.13	0.20	0.015(11.6)
IMMUNE 5	120	0.60±0.01	0.05	0.03	0.36	0.99	5.15	14.32	4.32	0.17	0.028(25.3)

[#]   Mice immunized orally against S.adelaide (IMMUNE), V.cholerae (CONTROL) or nonimmunised (NORMAL)

[*]   Of 471,700 cpm (500ng) I^{125}FLA fed.
Liver unperfused; small intestine rinsed; carcass after removal of above organs;
blood 0.13 ml undialysed; plasma from 0.13 ml blood after dialysis (figures in parenthesis
indicate % non-dialysability of label in plasma).

Table 7.5   Absorption of H^3DNP-labelled antigens in the intact mouse

Exp	H^3DNP antigen	Dose fed cpm[#]	mg	Time of kill (h)	Status of mice[+]	RECOVERY (%DOSE±S.E.) liver[*]	Plasma[@]	P (liver)
1	FLA	6.35	0.8	0.5	normal	7.50±0.42	0.071 (0.066)	0.040
					immune	5.96±0.62	0.037 (0.024)	
2	FLA	6.35	0.8	1.0	normal	4.93±0.39	0.009	0.138
					immune	3.19±0.59	0.007	
3	BA	6.35	0.4	0.5	normal	0.66±0.09	0.011	0.040
					immune	0.33±0.11	0.009	

[#] $\times 10^{-5}$

[+] Immune mice were immunized orally with live V.cholerae or S.adelaide

[*] perfused, undialysed

[@] Obtained from 3x0.13 ml blood and dialysed; figures in parenthesis indicate results from repeat experiment.

(3 mice / group)

again, this difference was more significant early on. The difference between the non-dialysable radioactivity in the plasma of normal and immune mice for either antigen was less consistent or meaningful, though it appeared that there was more radioactivity in the immune plasma.

7.1.4   Using H^{3}DNP antigens in vivo

Findings similar to those obtained in 7.1.3 (using I^{125} antigens) were also obtained in the intact mouse using H^{3}DNP-BA and H^{3}DNP-FLA (Table 7.5). Thus, there was significantly less uptake of either antigen in the immune mouse as measured by the total radioactivity in the liver; furthermore, the non-dialysable radioactivity in the plasma was also less in the immune animal, which was particularly marked for FLA at 1/2 h.

7.2   The Site of Coproantibody Action

It was seen in 7.1.3 that the small intestine of immune mice, after being washed extensively with a jet of running tap water, contained significantly more antigen (I^{125} label) than that of normal mice. For example, about 1.5x more I^{125}BA was recovered in the immune intestine at 1/2 h post feeding (P=0.0039; Fig. 7.1). It is likely that this figure truly represents BA since > 70% of the radioactivity in the homogenised intestine was high molecular-weight. The enhanced uptake of the specific I^{125} antigen by the intestine of actively, orally immunized mice was also observed in Fig. 6.1B and in Table 7.6. In fact, this could be mimicked by feeding pre-formed immune-complexes (using mouse antiserum or mouse immune intestinal juice) to normal mice (Table

Table 7.6A   Enhanced uptake of $I^{125}BA$ by the immune intestine in vivo: studies on the rinsed intestine

Exp	Time of kill (h)	No. mice	Label adsorbed to rinsed gut (%DOSE ±S.E.) NORMAL	IMMUNE	P
EF[#]	1.5	5	2.83 ± 0.36	5.90 ± 0.35	0.0290
JK[@]	2.0	4	2.68 ± 0.45	4.08 ± 0.15	0.0125
MN[*]	0.5	4	7.17 ± 0.31	13.62 ± 0.55	0.0125

[#] NORMAL and IMMUNE mice were fed 1.57×10^6 cpm $I^{125}BA$ in saline

[@] Normal mice were fed 1.66×10^6 cpm $I^{125}BA$ that had been incubated at R.T. for 10 min with 0.02 ml mouse anti-BA (#1276)(IMMUNE) or normal mouse serum (NORMAL)

[*] Normal mice were fed 1.47×10^6 cpm $I^{125}5BA$ that had been incubated at R.T. for 10 min with 0.05 ml IIJ (IMMUNE) or NIJ (NORMAL)

Table 7.6B   Enhanced uptake of $I^{125}BA$ by the immune everted gut sac

	RECOVERY OF RADIOACTIVITY[@] ± S.E.			
	jejunum		ileum	
	NORMAL	IMMUNE	NORMAL	IMMUNE
sac wall	43.0±4.9	51.8±10.3	46.5±5.3	70.9±2.7
rinse	22.9±3.7	32.6±2.9	15.0±1.3	15.8±0.6

[*] 4-5 sacs / group were incubated in 175,000 cpm/ml $I^{125}BA$ for 1 h at $37^{o}C$. They were rinsed lightly once and the contents were withdrawn; the rinse and sac wall were counted

[@] (Total cpm present ÷ cpm in 0.5 ml bath)%

Table 7.6C Enhanced uptake of $I^{125}BA$ by the immune intestine in vivo: studies on the rinsed intestine treated with DTT*

| | radioactivity adsorbed to gut (%dose ± S.E.) | | | |
| | T R E A T M E N T   O F   A N T I G E N | | | |
	saline	IIJ	DIgM	RAB
UNTREATED	7.40±0.59	8.33±0.81	8.45±0.84	7.02±0.42
DTT treated	0.86±0.21[@]	2.00±0.18[@]	1.53±0.06	(not done)

* Groups of 5 mice were fed 600,000 cpm $I^{125}BA$ that had been pre-incubated at R.T. for 30 min with 0.05 ml IIJ, 0.02 ml mouse anti-BA (DIgM), 0.02 ml rabbit anti-BA (RAB) or saline. After 30 min, the mice were killed and the small intestines were excised, rinsed and counted (UNTREATED). The intestines were then incubated in 1 mm DDT at $37^{o}C$ for 15 min, rinsed and counted again.

[@] P=0.0164

Table 7.6D Enhanced uptake of $I^{125}BA$ by the immune intestine in vivo: studies on the mucosal extract*

| | RADIOACTIVITY RECOVERED (%DOSE± S.E.) | |
	normal mice	immune mice
Mucosal extract	1.13 ±0.16	2.33 ± 0.21
P		0.0125

* Groups of 5 mice were fed 300,000 cpm $I^{125}BA$ and then killed 30 min later   The isolated small intestine was gently rinsed with 2 ml cold PBS-1% BSA. The rinse was discarded. The intestine was massaged firmly and a further 2 ml of buffer was passed through, massaging at the same time. This rinse (mucosal extract) was collecte and counted. It was clarified and the supernatant obtained was treated with 50% sat.ammonium sulphate. There was no precipitation in both NORMAL and IMMUNE.

7.6A). This effect was also seen in everted gut sacs that were only lightly rinsed once (Table 7.6B) (unlike the more vigorous rinsing used in other gut sac studies).

These observations suggest that antibodies complex with the antigen at the mucosal surface (rather than in the lumen) to prevent antigen uptake. The complex is probably held loosely in the mucus. In support of this, it was found that 80-90% of the adsorbed radioactivity in the washed intestine of mice fed with either I^{125} BA or I^{125} BA-antibody complexes, could be removed using DTT, a mucolytic agent (Table 7.6C). (In this study, the residual radioactivity in the intestine after treatment with DTT was still greater in mice fed the complexes than those given the antigen alone; whether this is due to incomplete removal of the mucus (and complexes) is not known.) The concommitant removal of both mucus and the inhibitory function of coproantibodies by DTT was also seen in everted gut sac studies, discussed in 7.1.2.

A similar finding that the immune intestine adsorbed more radioactivity than the normal intestine, was also obtained in the experiment recorded in Table 7.6D. Here, an attempt was made to detect immune-complexes in the immune mucosa. The mucosal extract obtained from these mice (by massaging the intestine) was clarified, and the supernatant was precipitated with 50% saturated ammonium sulphate (without extraneous antibody). There was no evidence of pre-formed immune-complexes in the extract.

7.3   The Induction of Intestinal Anaphylaxis

7.3.1   In germ-free mice in vivo

An attempt was made to compare the relative efficiencies of antigen blockade by immune intestinal juice (sIgA) and serum antibodies (IgM or IgG). Thus, equivalent amounts (according to the Farr titre) of mouse anti-BA intestinal juice (IIJ) or mouse antiserum (0.2 ml total of MAB #DIgM) were mixed with the antigen (31 mg BA) and fed to normal germ-free mice in a schedule described in 7.1.1. While mice fed with IIJ showed a decreased uptake of the antigen compared with those given NIJ (discussed in 7.1.1), and both groups of mice were healthy after the 4 antigen doses, the 5 mice fed with the antigen-antiserum mixtures died within 45 min of the third antigen dose. The intestines of these mice were haemorrhagic and inflamed, unlike those of other groups.

It thus appeared possible that the mice fed with antigen-antiserum mixtures had died of intestinal anaphylaxis, as shown by the macroscopic appearance of the intestine. The involvement of the intestine was further investigated using an _in vivo_ approach discussed in 3.3.2. Here, the transport from the circulation into the gut of two i.v.-injected markers (I^{125} BSA and Pontamine Sky Blue) unrelated to the sensitizing antigen, was used to assess the gut permeability and damage. Using this technique, it was found that more I^{125} BSA and dye leaked into the intestine with increasing amounts of antiserum (MAB #1276) used in the oral mixtures (Table 7.7). Antisera from other sources (#LB; #DIgM - used previously) could also mediate it but not normal mouse serum.

Table 7.7   Intestinal permeability studies in germ-free mice

| ORAL   CHALLENGE | INTESTINAL   WASH-OUT | |
	radioactivity (% dose)	Presence of dye
Saline*	0.21	-
Saline	0.17	-
0.20 ml normal mouse serum	0.17	-
0.03 ml MAB # 1276	0.30	-
0.07 ml MAB # 1276	1.23	+
0.20 ml MAB # 1276 @	1.81	++
0.20 ml MAB # LB	0.55	+

* no antigen

@ mouse later died

Mice were fed half-hourly with 3 x 0.5 ml BA # 878 (20 mg total) mixed with saline, normal serum or mouse antiserum (MAB) (total vol indicated). Half-hour after the last dose, 0.2 ml I^{125} BSA (ca. 175,000 cpm) in 1 % Pontamine Sky Blue was injected i.v. and half-hour later, 3 ml saline wash-out of the small intestine was obtained.

Gut permeability was investigated further. For example, it was obviously of importance to see if IgA could prevent this effect. However, in order to study this, a more sensitive system using less antigen and sensitizing antibody was necessary.

In a preliminary study, it was found that 6 mg BA (#878) and 0.075 ml antiserum (#1276) given as 2 half-hourly doses produced measurably increased gut permeability. Thus, $0.32 \pm 0.03\%$ radioactivity was recovered in the intestine when 140,000 cpm I^{125} BSA was injected i.v. (1/2 h after the second dose) and the mouse was sacrificed 1/2 h later. ($0.13 \pm 0.01\%$ was recovered in control mice fed with the antigen only). Using this system, the effect of immune intestinal juice was investigated. BA-antiserum mixtures were incubated with IIJ or NIJ (5 vol IJ : 1 vol antiserum) for 15 min/room temperature, and fed to normal germ-free mice. The amount of I^{125} BSA transported to the gut was then assayed. The results obtained with IIJ ($0.25 \pm 0.07\%$) and NIJ ($0.28 \pm 0.05\%$) in groups of 6 mice were not significantly different (P=0.4725).

A shortage of germ-free mice unfortunately prevented further studies in these mice; however, observations were continued in SPF mice and these are discussed in the next section.

7.3.2   In SPF mice in vivo

Normal SPF mice when given the antigen-antiserum mixtures (31 mg BA; 0.2 ml MAB#1276) that were toxic to germ-free mice (7.3.1), did not die,* but showed an increased gut permeability. (Even the latter was more variable and less easily produced; see below.)

*for reasons not entirely clear

It is possible that the lack of modulating effect of immune
intestinal juice on the permeability phenomenon above could be
partly due to the high proteolytic activity of the juice which
would degrade the 'anaphylactic' serum antibody. Since it is
difficult to obtain large amounts of purified, specific IgA,
another model was studied. The question of whether the sIgA
predominantly produced in the gut of orally immunized mice would
influence the adverse effect resulting from the feeding of
antigen-antibody mixtures as seen in normal mice, was thus
examined. Contrary to expectation, the immune mice not only
showed increased permeability when given the toxic mixture, but
also when the antigen was given alone (P=0.0238) (Table 7.8, Exp
#1). In the same study, the gut permeability of normal mice was
only increased (P=0.0125) when the BA was fed with specific
antiserum. Similar observations were obtained in Exp #2. Thus, a
greater transport of circulating I^{125} BSA to the intestine of
immune mice was seen when the animals were challenged with the
specific antigen (BA), mixed with NIJ or normal mouse serum,
although normal mice given the same BA-NIJ mixture showed little
effect. Interestingly, this effect was reduced significantly
(P=0.0402) when the immune mice were fed BA-IIJ instead,
suggesting perhaps a modulation by sIgA. (The IIJ was previously
incubated at $37^{\circ}C/1$ h to remove intact IgM and IgG; Horsfall et
al 1978.)

The increased non-specific permeability of the immune intestine
following challenge with the specific antigen was also seen using
S.adelaide LPS as antigen (Exp #3; P=0.0402).

Table 7.8   Intestinal permeability studies in SPF mice[#]

Exp	Group	MICE Status[*]	No.	Oral Challenge[@]	Intestinal wash-out (%DOSE±S.E.)	P (Groups)
1	1	normal	4	BA + NMS	0.31 ± 0.02	
	2	normal	4	BA + A/S	0.70 ± 0.08	0.013 (1&2)
	3	immune	4	BA + NMS	0.82 ± 0.21	0.024 (1&3)
	4	immune	4	BA + A/S	1.10 ± 0.26	
2	1	normal	5	saline	0.32 ± 0.10	
	2	normal	5	BA + NIJ	0.37 ± 0.13	
	3	immune	3	Saline	0.29 ± 0.03	
	4	immune	3	BA + NIJ	1.42 ± 0.07	
	5	immune	6	BA + IIJ	0.90 ± 0.08	0.040 (4&5)
	6	immune	3	BA + NMS	1.42 ± 0.17	
3	1	normal	3	LPS	0.23 ± 0.05	
	2	normal	3	saline	0.36 ± 0.10	
	3	immune	3	LPS	0.73 ± 0.12	0.040 (1&3)

[#] Protocol as in Table 7.7 except that challenge was given as 2 half-hourly doses; ca. 190,000 cpm I^{125} BSA was injected in all cases.

[*] Immune mice were immunized orally and i.p. with live V.cholerae (Exp.1&2) or live S.adelaide (Exp.3).

[@] Challenged with saline or the specific antigen: 4.4 mg (total) BA#878 (Exp.1), 3.2 mg BA#179 (Exp.2) or 6.0 mg S.adelaide LPS (Exp.3). BA was given with 0.125 ml normal mouse serum (NMS), 0.125 ml mouse antiserum (A/S #1276), 0.25 ml IIJ (preincubated at 37°C for 1 h) or NIJ. The mice were also prefed 5 min before each oral dose with 0.1 ml sat $KHCO_3$ (Exp 1) or 0.2 ml mixture containing 1:1 sat $KHCO_3$ and NIJ/IIJ (Exp 2).

7.3.3   In everted gut sacs

An attempt was made to study the intestinal anaphylaxis further in an in vitro system, where the influencing factors are conceivably more controllable and defined. Thus, everted gut sacs prepared from the ileum of normal SPF mice (4.6.8) were pre-incubated in Hanks medium (oxygenated) at $4^{o}C/18$ h to remove any mucus, enzymes or endogenous immunoglobulins (using the pre-treatment approach used in the Schultze-Dale experiment; Kabat et al 1963). They were then sensitized with either normal mouse serum (control) or mouse anti- S. adelaide serum, and later incubated in Hanks medium containing the specific antigen, S.adelaide LPS (6 mg/ml) and I^{125} BSA. After 1 h, the sac contents were removed, dialysed and counted. As seen from Fig. 7.2, the transport of the unrelated marker, I^{125} BSA, was significantly greater (P=0.0406) in those sacs sensitized with the specific antiserum than in control sacs treated with normal mouse serum.

7.4   Discussion

The results presented here confirm the findings of others (Walker et al 1972a; Andre et al 1974; Swarbrick et al 1979) that oral immunization inhibits the absorption of specific antigens from the gut into the circulation. This was observed in four different models:

(1) In the in vivo model using I^{125} specific antibody to detect antigen, immune intestinal juice passively given with BA reduced the amount of antigen absorbed to below detection level. This effect is remarkable since the amount of IIJ used (0.6 ml total)

Fig. 7.2

Intestinal permeability studies *in vitro*

Everted gut sacs prepared from the ileum of normal SPF mice were
pre-incubated in Hanks medium for 18 h/4°C. Groups of 6 treated
sacs were then sensitized with either mouse anti-*S. adelaide* serum
or normal mouse serum (37°C/90 min). These sacs were later placed
in Hanks medium[*] (pH 7.5) containing *S. adelaide* LPS (6 mg/ml) and
I^{125}-BSA (27,700 cpm/ml). After incubation for 1 h/37°C, the sac
contents were removed, dialysed and counted.

*   also contained 4% guinea-pig serum and 0.4% cold BSA.

Fig. 7·2
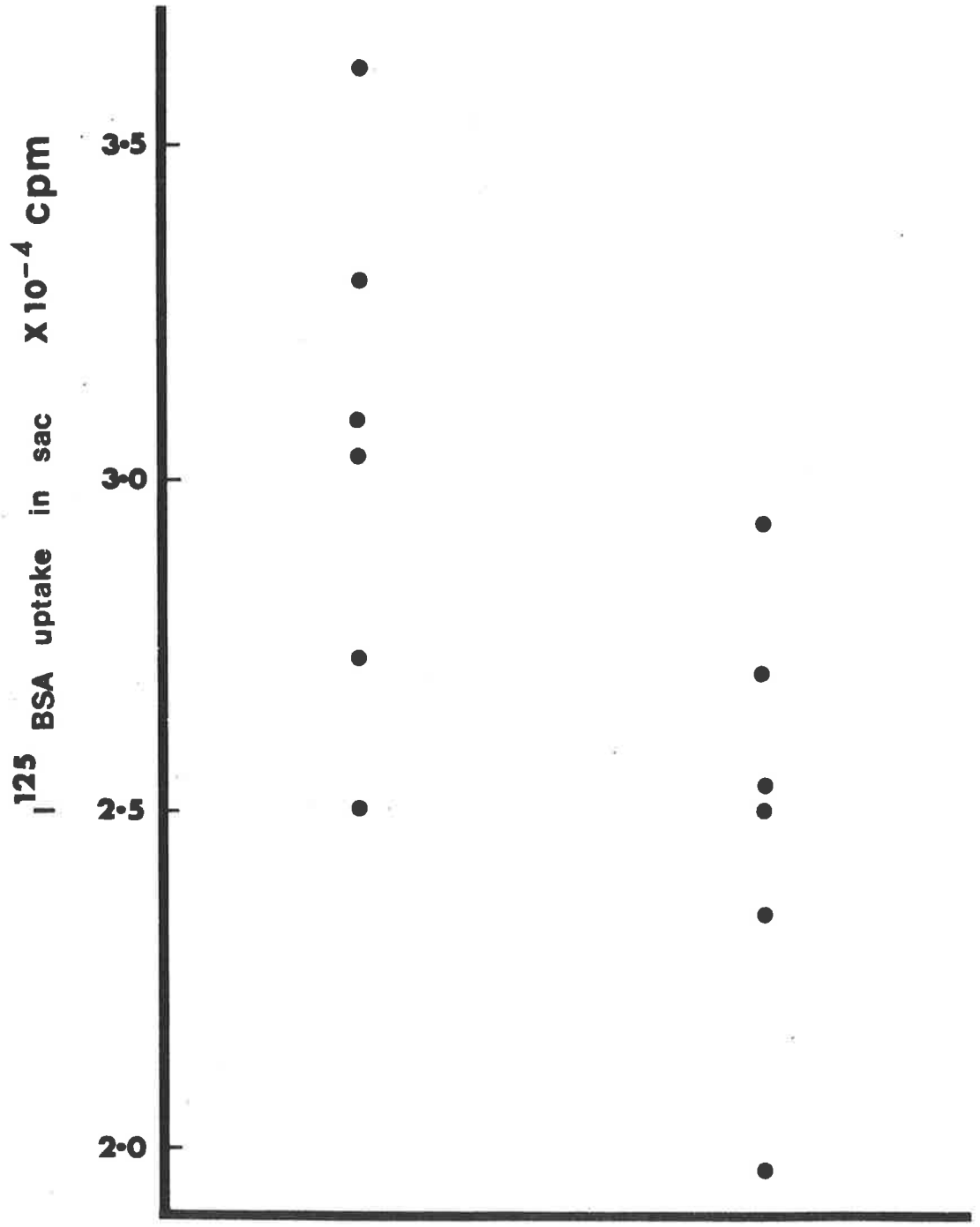
sac sensitized with anti-LPS

sac sensitized with normal serum

Mean ± S.E.   30,480 ± 1,620 cpm   24,910 ± 1,360 cpm

is small in relation to the amount of antigen fed (31 mg), and must only represent a fraction of the activity present in the immune gut of the intact animal. Implicated here is the direct inhibitory function of immune intestinal juice and presumably sIgA antibodies (since this class is most predominant; see Horsfall & Rowley 1979).

(2) Everted gut sacs from immune mice also showed decreased uptake of I^{125} BA. In terms of the non-dialysable radioactivity in the sac contents, 58% maximal reduction was observed after 1 h. Whether this rather low inhibition is due to the high sensitivity of the assay or to the dilution effect of inhibitory factors is not clear. Although the major site of antigen blockade (the jejunum or the ileum) was not ascertained in these studies, the whole intestine is probably involved, depending on where the coproantibodies are secreted or localized.

(3) It was discussed in 6.6 that the total radioactivity absorbed in the intact mouse fed with I^{125} antigens probably reflects the absorption rate of the antigen, even though no antigen-associated radioactivity could be found. Accepting this, immune mice absorbed less I^{125} BA and I^{125} FLA than normal animals, as measured by the total radioactivity in the internal organs, especially at early times, e.g. 29% and 13% reduction were observed in the liver uptake at 1/2 h for BA and FLA respectively.

(4) This was also observed in mice fed with the more stable conjugates, H^3 DNP-BA and H^3 DNP-FLA, as measured by the total radioactivity in the liver and the non-dialysable radioactivity in

the plasma. For instance with H^3 DNP-FLA, the reductions in the liver and plasma were 21% and 55% respectively. (In retrospect, it would be more appropriate to dialyse the liver before counting; nevertheless, the % dialysability of the radioactivity in extracts from normal and immune mice does not appear to be different.) The use of H^3 DNP-antigens is also attractive in these studies since, with the availability of an IgA myeloma (MOPC315) that has anti-DNP activity, the inhibition of macromolecular absorption by IgA could be quantitatively studied and compared for the different antibody classes. (Unfortunately, we have not used this approach since our H^3 DNP-preparations were poorly bound by MOPC315 as determined in a Farr assay.)

Coproantibodies probably exert their inhibitory effect by complexing antigens in the mucous layer (rather than in the lumen). This was first suggested by the finding that the washed intestine of immune mice fed with the specific I^{125} antigen had more radioactivity adsorbed than that of normal mice. This was supported by similar findings in everted gut sac studies, and also by the concommitant removal of both mucus and antibody activity by dithiothreitol (as also observed by Walker et al 1974a).

The reduced uptake into the circulation of specific antigens in immune animals is probably due to the immune exclusion of the antigen, rather than to the immune elimination of it from the circulation.[*] This is evidenced by the higher radioactivity recovered in the gastrointestinal tract of immune mice fed with I^{125} antigens, compared with that of normal mice (and correspondingly the lower absorption in these mice). This is also supported by the inability to detect pre-formed immune-complexes

[*] *See 7.6*

in the contents of everted gut sacs derived from orally immunized animals, using the Farr assay. However, this may not be a sensitive technique for detecting complexes, particularly with low-avid antibodies (e.g. coproantibodies ?) (see Fig. 4.2). This may also account for the failure to detect immune-complexes in the intestinal mucosa of immune mice fed with the I^{125} specific antigen (BA) using this technique. The detection of immune-complexes on sucrose density gradients (as successfully used by Walker et al 1975; Walker and Bloch 1977) may be more rewarding; however, this is not suitable for heterogenous antigens like BA.

If inhibition of antigen uptake is a desirable function, it seems that this may be normally carried out by IgA coproantibodies, since adverse effects resulted when serum antibodies were used. This effect was fatal as seen in germ-free mice fed with the immune-complexes, and also in the form of increased intestinal permeability to unrelated antigens in general. The latter phenomenon was also observed in both normal and immune, conventionalized mice when challenged with the BA-antiserum mixtures; the antigen alone could also mediate this effect in the immune animals and the implication of this is discussed in 9.5.2. More work is certainly required to explore the mechanisms of these reactions, with regards to the antibody classes involved, the requirement for exogenous complement, etc.[*] Further work is also necessary to elucidate the possible moderating influence of IgA on the phlogistic reactions of these antibody classes at the mucosal level. The few experiments attempted here to show this were rather unsuccessful, probably because the conditions of assay in these in vivo studies were not

[*]See 7.6

optimal. The difficulties of achieving optimal doses of sensitizing antigen and antibody, together with the problem of degradation of these reagents in the intact animal, may perhaps be minimized in the everted gut sac model described above (for studying intestinal anaphylaxis). Although this has not been done, it is envisaged that more fruitful results will be obtained from using this model together with MOPC315 as the blocking IgA anti-DNP antibody, and DNP-BSA as the sensitizing antigen.

7.5   Conclusions

Oral immunization reduced the intestinal absorption of the specific antigen (BA or FLA). The degree of inhibition depends on the sensitivity of the assay; this was less than 58% when radiolabelled antigens were used in both _in vivo_ and _in vitro_ models. The inhibitory function is probably one of immune exclusion, due to coproantibodies complexing with the antigen in the mucous layer. This may be normally carried out by IgA antibodies since serum antibodies (IgM or IgG) may induce intestinal anaphylaxis, as manifested by death or increased gut permeability.

7.6 Addendum: on the Mechanisms of Immune Exclusion and Intestinal Anaphylaxis

Both our studies and those of others (Walker et al 1974b; Tolo et al 1977) have shown that coproantibodies block antigen uptake by complexing with the antigen in the mucous layer or in the connective tissues. What happens then to the antigen remains speculative (see 9.5.2). However, it appears that the complexed antigen becomes more readily degradable *in situ* than the free antigen. Thus, Walker et al (1974b) observed enhanced degradation of I^{125}-BSA in the immune intestine by pancreatic enzymes, while Tolo et al (1977) ascribed this action to lysosomal hydrolases released from phagocytes. The latter assumption was based on the observation of Cardella et al (1974) that immune-complexes stimulated the release of these enzymes. Our finding that the dialysability of the radiolabel recovered from immune gut sacs was generally higher than that from normal sacs (Table 7.2) is consistent with their hypothesis.

Although the specific antigen is retarded and degraded in the presence of (IgG?) antibodies, the same process can cause tissue injury (Cardella et al 1974) and increased gut permeability to unrelated antigens (Brandtzaeg & Tolo 1977). Thus, these findings and ours suggest that immune-complexes formed at the mucosal level can directly induce altered permeability, perhaps as a consequence of lysosomal enzymes released. It must be nevertheless remembered that this phenomenon is rather complex, which may involve vascular permeability, intestinal epithelial permeability, mucosal haemodynamics etc. (see Nawa 1979). In other words, other causes can also bring about this permeability change e.g. disease (Jeffries et al 1962), lysis of mast cells by IgE-antigen complexes (Murray et al 1971), and toxicity or physical damage resulting from Nippostrongylus brasiliensis infection (Nawa 1979). Interestingly, the same approach was used in these other studies as that adopted by us i.e. by determining the recovery in the gut of

i.v.-administered markers (radioiodinated-albumin, -polyvinyl pyrrolidone or Evan's blue). In retrospect in our studies using I^{125}-markers, it may perhaps be more accurate to gauge permeability from the index, (cpm recovered in gut contents)/(cpm in 0.1 ml blood @ 5 min post injection), than from the radioactivity recovered in the gut *per se*, and to follow the kinetics of recovery.

8.   CHAPTER 8. THE INTESTINAL ABSORPTION OF IMMUNOGEN IN ADULT

MICE

> "The appearance of circulating
> antibodies in the blood after
> oral administration of an
> antigen is an observation
> almost as old as immunology
> itself......However, the vast
> literature on the subject has
> cast little light with regard
> to the site of origin or the
> immunoglobulin classes of the
> antibodies formed in response
> to oral stimulation."
> (Heremans 1969)

The earliest indications that proteins are absorbed intact through the gastrointestinal tract are probably those relating systemic allergies to eating certain types of food (Brunner and Walzer 1928), and those where serum antibodies to antigens believed to gut-derived were found (Erhlich 1891; Uhlenhuth 1900; Coombs et al 1965; Triger et al 1972).

In all these studies however, the oral absorption of immunogenic material was only circumstantially inferred, and it is possible that some of these antibodies may be due to cross-reacting antigens. The direct recovery of absorbed immunogen (as opposed to antigen) has not been demonstrated in any in vivo study, although Nolan et al (1977) convincingly showed that immunogenic endotoxin was transported in everted gut sacs. Further, whether immunogenic material can be recovered from tissues important in antibody synthesis (e.g. the Peyer's patches, lymph node and spleen) is quite unknown. It is possible for instance, that the

systemic antibody response to oral antigens is derived solely from gut-associated lymphoid tissues without the stimulation of "systemic" tissues by free immunogen absorbed from the gut.

In this chapter, attempts to demonstrate the intestinal absorption of immunogenic BA in mice will be examined.

8.1   Establishing a Sensitive Immunogenic Assay for BA

The immunogenic potential of BA was exploited by utilising the ability of the test antigen to prime an animal, rather than to elicit antibody production per se (as discussed in 3.4). SPF mice were injected i.v. with 1-1000ng of BA in pyrogen-free saline and then boosted i.v. with 100ng of the same antigen 3-4 weeks later. The mice were bled 11 days after the priming dose and 4, 6, 9 and 11 days after the booster. The sera obtained were titrated by passive haemagglutination (4.6.2). The results for the primary and secondary (days 4 and 6) responses are shown in **Fig. 8.1**. Thus, mice primed with 100ng BA or more developed significant haemagglutinating antibodies in both the primary and secondary responses, while those primed with as little as 1ng (of this preparation), produced antibodies only on boosting. The booster given to nonimmunized mice (control group) did not stimulate any response. The anamnestic response emerged and subsided quickly, peaking at 4-7 days after the booster, and declining activities were observed in day 9 and 11 sera.

In the same study (Fig. 8.1), groups of mice were also primed by oral feedings with graded doses of BA (0.001 - 1 mg). They were similarly boosted with 100ng BA i.v. and their responses

Fig. 8.1

Immunogenic studies with BA#DBA.

Groups of 3 mice were primed with BA either orally in 20% $KHCO_3$ or $i.v.$
in pyrogen-free saline and bled 11 days later. These mice were challenged
4 weeks later with 100 ng BA $i.v.$ and bled on days 4 and 6 after the
booster. The sera obtained were titrated by haemagglutination (HA), and
results from individual mice are shown. The specific immune clearance of
these mice was tested on day 7 after the booster, using 30,000 cpm (0.1 μg)
I^{125}-BA injected $i.v.$ The phagocytic index (P.I.) was determined by the
rate of clearance from the circulation; the uptake by the unperfused
liver (Liver, % dose) was also measured after 15 min . (Results expressed
as average/group).

Fig. 8.1
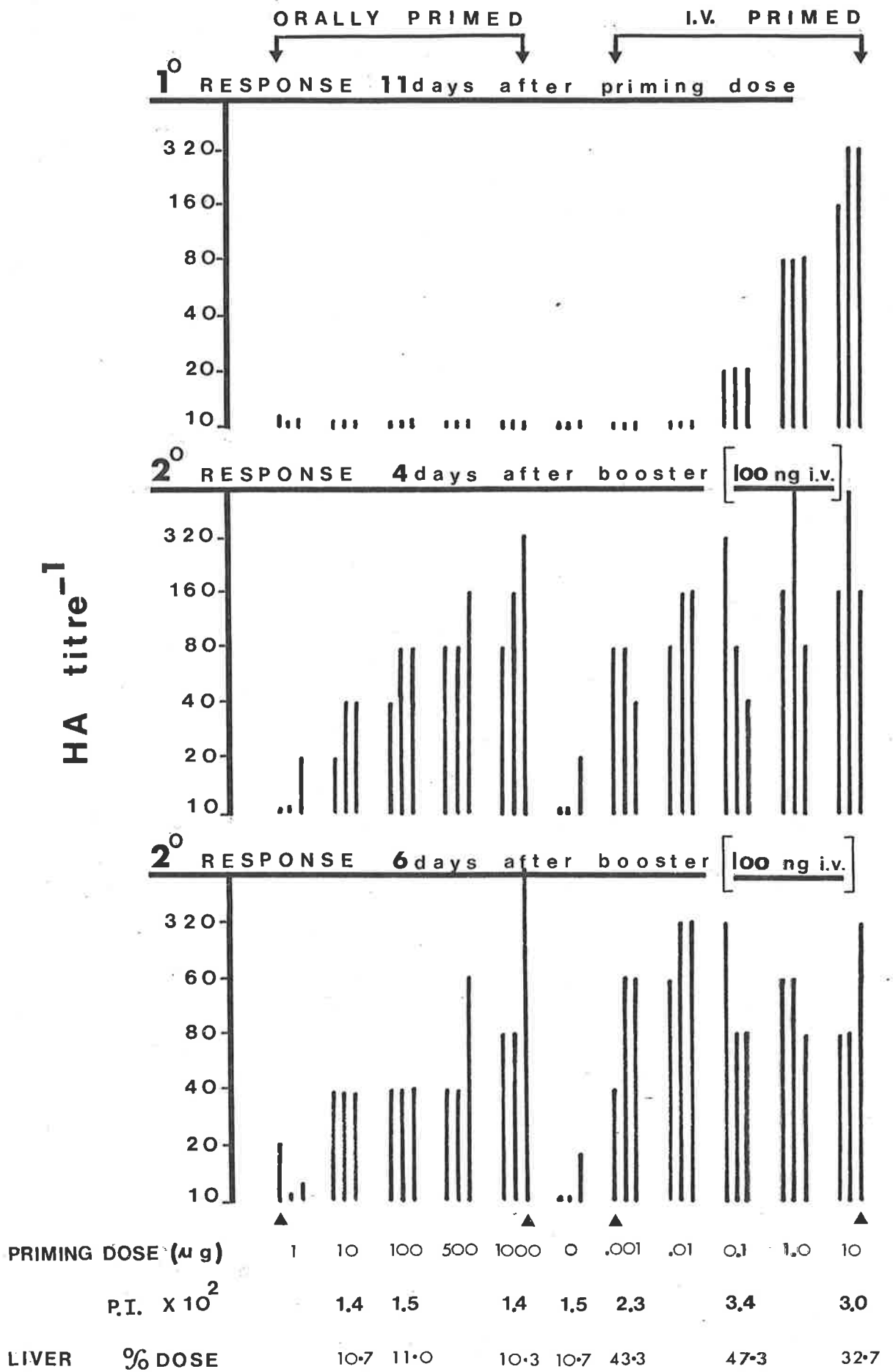
were determined in the same way. There was no direct response to the oral dose after 11 days in all groups, but haemagglutinating antibodies were found in those primed with more than 0.01mg following boosting.

The immune status of the mice was also judged by their ability to clear a dose of I^{125} BA injected i.v. 7 days after the booster. This was expressed as the conventional phagocytic index (P.I.), i.e. the rate of clearance of I^{125} BA from the circulation between 0-15 min post injection (see Jenkin & Rowley 1961), and as the total radioactivity in the unperfused liver after 15 min. Thus, all mice primed intravenously that possessed haemagglutinating antibodies, also showed good clearances. For instance, in the 100ng group, P.I.=0.034 and the liver uptake was 47.3% of the dose injected, compared with 0.015 and 10.7% respectively for the control group. However, the surprising finding was that the orally-primed mice did not clear the I^{125} BA, despite demonstrable haemagglutinating antibodies in their sera (e.g. P.I. = 0.014 and liver uptake = 10.3% for the 1 mg-group). It was also found that the day 6 sera of these mice (0.1mg oral) was not opsonic when injected with I^{125} BA into normal mice, whereas sera from the 0.01mg i.v. primed group (with equivalent haemagglutinating titres) were opsonic (Table 8.1).

The lack of opsonic activity in the sera of the orally-primed animals may be due to IgA antibodies. Attempts to demonstrate these were made using the radioimmunoassay technique described in 4.6.5. However, only trace amounts of antibody were detected with the I^{125} goat anti-mouse IgA, whereas activity could be easily detected in both the orally-and i.v.-primed groups using I^{125}

Table 8.1   Characterization of day 7 sera from orally and i.v. primed mice[+]

PRIMING GROUP	RIA - LC titre	%bound[@]	RIA - IgA titre	%bound[#]	LIVER[*] (% DOSE) 10µl AS	50µl AS
1.0 mg oral	>1:640	9.38	1:40	5.53	9.0	10.2
10 µg i.v.	1:640	8.50	1.20	4.88	11.1	14.3
Saline Control	1:40	5.50	1.20	4.68	8.8	ND

[@] % radioactivity bound using 1.25 µl test serum (1:80 dilution) and 8000 cpm (3.1ng) I^{125} goat anti-mouse IgLC

[#] %radioactivity bound using 2.5 µl test serum (1:40 dilution) and 8000 cpm (2.7 ng) I^{125} goat anti-mouse IgA

[*] opsonic test: 10 or 50 µl test serum was incubated with 60,000 cpm (0.2µg) or 30,000 cpm (40ng) 1^{125} BA respectively at 37°C for 1 h and then injected i.v. into normal mice.   The radioactivity in the unperfused liver after 15 min was determined.

[+] sera pooled from 3 mice in each group and used - see Fig. 8.1

goat anti-mouse IgLC (Table 8.1).

8.2   Preliminary Immunogenic Studies with FLA

The _in vivo_ immunogenic assay used for BA above was also attempted with FLA (flagellin) over a wide range of i.v. doses (up to 100ng) for both the priming and booster doses. However, there was no activity detectable by haemagglutination in any of these sera. In other words, this antigen is less immunogenic than BA under these circumstances. This is rather surprising in view of the good immunogenicity of FLA reported by Nossal _et al_ (1964) in rats. The relatively poor immunogenicity of FLA in this system, and the relative scarcity of it (large amounts are required for oral studies) discouraged further studies with this antigen in this area.

8.3   Further Immunogenic Studies with BA

The experiments discussed in 8.1 show that orally-administered BA has the ability to prime mice for a subsequent humoral response. Further, the ensuing response is different from that obtained by i.v.-priming. Namely, although haemagglutinating antibodies are produced in both groups after the booster, those from the orally -primed groups do not appear to be opsonic, perhaps indicating an IgA response. Consequently, further experiments were conducted to probe the questions: (1) what is the basis of this difference in the inductive mechanisms between i.v.- and orally-primed mice; and (2) can immunogenic material be recovered directly from the circulation and tissues of mice fed with the antigen?

The results of these experiments are shown in Tables 8.2 - 8.4. The experimental approach, (based on the immunogenic assay described in 8.1), was as follows: Groups of 3-6 mice were primed orally (OR) with BA in 20% bicarbonate, or intravenously (IV) with the antigen in pyrogen-free saline, or intravenously (IV) with biological extracts derived from mice fed with BA. After 3-4 weeks, the mice were boosted with 100ng BA in saline i.v. They were bled 4-6 days after the booster, while their specific immune clearance of the antigen was determined on days 5-7. In the clearance test, the amount of radioactivity in the unperfused liver (L) and 0.13 ml blood (B) was determined 15 min after the i.v. injection of I^{125} BA; the "LB" quotient (L cpm/B cpm) ratio was used to gauge the phagocytic function, which was simpler and more sensitive than the tests used in 8.1 (see 3.3.1). The sera obtained were titrated by haemagglutination (HA) and by radioimmunoassay (4.6.5) using I^{125} goat anti-mouse IgLC (RIA-LC) or I^{125} goat anti-mouse IgA (RIA-IgA). Although these sera were initially examined by the standard RIA technique, a modification of the assay was used, owing to the poor binding (low avidities?) of these sera (see 4.6.5 & 8.4). Thus, the number of counts bound by 2.5µl test serum at a final dilution of 1:40 was determined. The effect of some of the sera in a standard opsonic assay (using serum MAB # 1276 and I^{125} BA) was also studied to see if they were inhibitory (due to IgA antibodies) or enhancing (due to IgG/IgM antibodies), rather than simply determining whether they were opsonic per se. This effect (denoted by "lb" in Tables 8.3 and 8.4) was determined from the "LB" in normal mice injected i.v. with these mixtures.

8.3.1   Immunogenic studies with BA # DBA

The salient features of this study as shown in Table 8.2 are:
(1) Extracts from the Peyer's patches of a mouse fed with 4 x 9 mg
half-hourly doses of BA (and sacrificed 1/2 h after the last dose)
were immunogenic (Group D). The mice injected with the extracts
developed high tires of antibodies (HA > 1:320) and low clearance
rates (LB = 5.5 ± 0.6). On the other hand, mice injected with
extracts from an equivalent mass of non-Peyer's patch tissue
(Group E) had very good clearances of the antigen (LB = 8.6 ± 1.5)
despite lower levels of haemagglutinating antibodies (1:80).
There was no activity in the Peyer's patches of a mouse fed with
the same amount of BA in the same schedule, but sacrificed 24 h
after the last dose (Group C).
(2) All mice that were orally-primed (Groups F - H) produced
detectable amounts of antibodies but showed no immune clearance of
the antigen. In contrast, the i.v.-primed mice (Groups I & J)
with equivalent or lower antibody levels had good clearances.

8.3.2   Immunogenic studies with BA # 878

Attempts were made in this study to recover immunogenic BA from
the circulation and tissues of groups of 6 mice fed with BA (mixed
with a trace of H^3DNP-BA), at various times post feeding.   The
salient features of this study (Table 8.3) are:
(1) The dialysed plasma of mice fed with BA was found to have
immunogenic activity half-an-hour later (Group M)_ and reached a
maximum after 1 h (Group N). No activity was found in the 2 h and
3 h samples. This is consistent with the antigenic concentrations
found in these samples (estimated from the non-dialysable

Notes on some PRIMING GROUPS in Table 8.2

Group A .... Control

Group D .... Primed with extracts[*] of the Peyer' patches (see 4.7.7
                 & 4.7.9) from a mouse fed half-hourly with 4 x 9 mg
                 BA#578 and killed 45 min after the last dose.

Group E .... Primed with extracts of an equivalent mass of non-Peyer's
                 patch tissues from the same intestine used in Group D.

Group C .... As in Group D, but the Peyer's patches were obtained from
                 a mouse killed 24 h after the last antigen dose.

Group B .... As in Group D, but the Peyer's patches were obtained from
                 a mouse given saline only.

Group H .... Fed with 5.1 mg BA#DBA on day 1, followed by 7 x 0.1 mg
                 oral feedings on days 3, 6, 9, 12, 16, 19 and 21. The
                 $i.v.$ booster was given on day 28 (like in all other
                 groups, using 0.1 µg BA#DBA).

Group J .... Each mouse was injected $i.v.$ with 8 x 22 µg BA#DBA using
                 the same schedule as in Group H.

Groups F, G .... Fed with the native antigen (BA#DBA) once only.

*   concentrated, dialysed supernatant, divided equally among 3 mice
    (0.2 ml each).

Table 8.2   Immunogenic Studies with BA # 578 and # DBA

PRIMING GROUP			LB[1]	HA[@]	RIA-LC[+]	RIA-IgA[X]
A. normal plasma	(IV)		ND	20	15.2	3.91
B. normal Pp		(IV)	3.1±0.3	20	ND	ND
C. 24 h Pp		(IV)	2.8±0.1	20	14.9*	ND
D. 2 h Pp		(IV)	5.5±0.6	>320	37.0*	ND
E. 2 h nP		(IV)	8.6±1.5	80	20.2*	ND
F. 0.5 mg	BA	(OR)	3.1	80	ND	ND
G. 1.0 mg	BA	(OR)	2.6±0.1	160	26.0	6.44
H. 5.1 +						
7x0.1 mg	BA	(OR)	2.7	80	18.4	4.13
I. 0.1 µg	BA	(IV)	6.1±1.2	80-320	18.6	4.43
J. 8x22 µg	BA	(IV)	6.6±0.4	>320	ND	ND

ND = not done   IV = intravenous priming   OR = oral priming

[1] Immune clearance : liver cpm / 0.13 ml blood cpm, determined 15 min post i.v. injection of 32,000 cpm (40ng) I^{125} BA, 7 days after booster.

[@] Haemagglutination titre (reciprocal) of serum obtained 4 days after booster.

[+] % radioactivity bound by 2.5 µl test serum (1:40 dilution) using either 16,760 cpm (12.5 ng) or 6,400 cpm (3 ng) (marked *) I^{125} goat anti-mouse IgLC.

[X] % radioactivity bound by 2.5 µl test serum using 16,800 cpm (8.ng) I^{125} goat anti-mouse IgA

(Results from 3 mice/group)

Notes on some PRIMING GROUPS in Table 8.3

The biological extracts used for priming were obtained from 4 groups of
6 mice; each group was fed a toal of 30 mg BA#878, containing a trace
of H^3-DNP-BA (*ca.* 70 cpm/μg, final specific activity). The mice were
bled and killed at ½, 1, 2 and 3 h post feeding. The different tissues
were extracted as detailed in 4.7.9 and the supernatants were used.

Groups M, N ... Primed with the dialysed plasma* obtained at ½ h and
       1 h post feeding respectively.

Group O .... Primed with extracts of the Peyer's patches (see Table 8.2)
       obtained ½ - 3 h post feeding. The antigenic dose
       injected per mouse (estimated from the non-dialysable
       radioactivity) was 180, 276, 256 and <100 ng for the
       ½ - 3 h samples respectively. (Selected from responding
       mice that had HA titres of 1:40 - 1:160).

Group P .... As in Group O, but with non-Peyer's patch extracts. The
       antigenic dose injected per mouse was 816, 1152, 126 and
       <100 ng respectively.

Group Q .... Primed with extracts of liver, spleen or mesenteric lymph
       node. For each organ, the supernatants were pooled from
       the 4 time-groups, concentrated and dialysed before use.
       (The results shown are averaged for mice in all groups).

Group L .... Primed with the native antigen, BA#878.

* from 2.2 ml blood (total) diluted in 3 ml Hanks medium containing
  10 U/ml heparin; 3-tenths of the plasma obtained was injected into
  each of 3 mice.

(All mice were boosted *i.v.* with 0.1μg BA#878 3 weeks after the
  priming dose.)

Table 8.3   Immunogenic studies with BA # 878

PRIMING GROUP	LB	HA_4	HA_6	RIA-LC	RIA-IgA	lb_4*	lb_6*
K. normal plasma (IV)	3.0±0.3	<20	<20	17.9±0.1	3.55±0.32	5.33±0.13	6.10±0.15
L. 188 ng BA (IV)	34.6±5.6	80-160	80-160	21.8±6.3	4.91±0.62	6.39	ND
M. 0.5 h plasma (IV)	ND	40-80	ND	22.1±4.5	ND	ND	ND
N. 1.0 h plasma (IV)							
mouse 1	2.8	160	160	24.7	7.93	3.77	3.17
mouse 2	3.0	80	20	21.0	4.91	4.36	5.94
mouse 3	3.2	80	20	19.9	3.20	ND	ND
O. 0.5-3 h Pp (IV)	12.3±1.4	40-160	ND	ND	6.03±0.40	ND	ND
P. 0.5-3 h nP (IV)	20.1±2.1	40-160	ND	ND	5.52±1.30	ND	ND
Q. organs (IV)	3.2±0.2	<20	<20	16.1±0.8	ND	5.46	ND

Legends as in Table 8.2; LB determined 7 days after booster using
113,800 cpm (100 ng) I^{125} BA; day 4 and 6 sera used in HA and lb,
while day 4 sera only were used in the RIA.

* Effect on standard opsonic assay : 50 µl test serum was incubated
with 44,750 cpm (0.4 µg) I^{125} BA at 25°C for 15 min. 25 µl opsonic
serum (MAB#1276) was then added.   After 5 min, the mixture was injected
i.v. into normal mice and the LB (=lb) was determined after 15 min.

(Results expressed as mean ± S.E. for 3-5 mice or, as in Group N, for individual mice).

radioactivity), which were 689, 608, 312 and 328 ng (amounts injected per mouse) for the 1/2 - 3 h samples respectively. However, the responding mice were unable to clear the antigen, unlike those primed i.v. with the native antigen that had similar haemagglutinating titres (Group L). That IgA was probably responsible for this, was suggested by the RIA - IgA and the inhibition of opsonic assay ("lb"), especially in mouse #1, Group N.

(2) Immunogenic activity was present in the Peyer's patches (Group O) and non-Peyer's patch tissues (Group P) between 1/2 and 2 h post feeding, but activity was lost in both at 3 h. Further, the activity appeared to be maximal at 1 h for both. This is consistent with the antigenic concentrations found in these extracts, determined from the non-dialysable radioactivity (see Table 8.3, footnotes). Both extracts conferred specific immune clearance in recipient mice, although this was somewhat less in those primed with extracts from the Peyer's patches.

(3) No immunogenic activity (or non-dialysable radioactivity) was found in extracts obtained from the liver, mesenteric lymph node or spleen (Group Q). That is, less than 0.00025% immunogenic BA of the dose fed was absorbed by any of these organs that could be extracted by the procedures used.

8.3.3   Immunogenic studies with BA # 179

The observation that immunogenic BA could be recovered from the plasma of mice fed with the antigen 1/2 - 1 h previously, but that it was different from the native antigen, warranted further investigation. The results in the present study confirm this finding (Table 8.4):

Notes on some PRIMING GROUPS in Table 8.4

Group S .... Primed with dialysed plasma* obtained from 20 mice that
                had been fed a total of 71.5 mg BA#179 1 h previously.

Group T .... Primed with the concentrated, dialysed contents pooled
                from 5 everted ileal sacs, which were incubated for 1 h
                in 0.65 mg/ml BA#179. (A trace of I^{125}-BA was included:
                final specific activity = 280 cpm/μg).

Groups U, V .... Primed with the native antigen, BA#179. (Selected from
                mice that had HA titres of 1:40 - 1:80).

* from 14 ml blood (total) diluted in 4 ml Hanks medium containing
  20 U/ml heparin; 10 ml plasma was obtained and concentrated to
  about 1 ml; this was divided equally among 5 mice.

(All mice were boosted *i.v.* with 0.1 μg BA#179 3 weeks after the
priming dose).

Table 8.4   Immunogenic studies with BA # 179

PRIMING GROUP	no. mice	LB	HA	RIA-LC	RIA-LC@	RIA-LC*	RIA-IgA	lb
R. normal plasma(IV)	3	3.2±0.1	<20	14.1±1.6	8.7±0.6	15.3±0.6	4.92±1.46	7.80±0.37
S. 1 h plasma(IV)	5	3.3±0.2	40	19.2±0.4	13.1±1.1	19.5±1.3	7.76±0.36	6.74±0.42
T. sac contents (IV)	6	3.4±0.2	40-80	20.2±1.6	19.6±3.9	19.1±0.6	7.85±1.10	6.57±0.18
U. 72-360 ng BA (IV)	4	10.5±2.1	40-80	17.2±1.3	13.2±1.6	19.5±2.4	4.98±0.64	8.83±0.86
V. 3 - 5 mg BA (OR)	3	3.5±0.1	40	16.1±2.0	9.5±1.4	15.9±1.9	4.58±0.06	6.41±0.46

Legends as in Tables 8.2 and 8.3; results expressed as mean ±S.E.
The sera used were obtained just before determining the LB (using
62,400 cpm / 0.6 µg I^{125} BA), all performed 5 days after the booster.

@ Using 2.0 µl serum and 9700 cpm (7.3 ng) I^{125} goat anti-mouse IgLC
* Using 2.5 µl serum and 11600cpm (8.7 ng) I^{125} goat anti-mouse IgLC

(1) The plasma was pooled from 20 mice which had been fed a total of 71.5 mg BA 1 h previously. This was used to prime 5 recipient mice for a subsequent antibody response. The serum from this group (S) had an average HA titre of 1:40 compared with <1:20 for the control group (R) primed with normal mouse plasma. This difference though small was consistent, and was confirmed by RIA-LC done on three separate occasions. However, these mice did not clear the I^{125} BA injected (LB = 3.3 $\pm$ 0.2), unlike mice that had been primed i.v. with the native antigen and had similar haemagglutinating activities (Group U). The presence of IgA in the plasma-primed mice was also suggested by the RIA-IgA and "1b" determinations.

(2) A further group of 6 mice were primed with the absorbed immunogen, obtained by dialysing the contents of everted ileal sacs which had been incubated in BA for 1 hour (Group T). This group developed significant responses, as measured by HA (1:40 – 1:80) and RIA-LC. However, despite the evident responses, these mice were unable to clear the antigen. This may have been due to the presence of IgA antibodies, as indicated by the RIA-IgA and "1b" values. Another interesting difference between the absorbed immunogen recovered from everted sacs and the native antigen is the apparent, diminished immunogenicity of the former. About 3 µg of absorbed antigen (estimated from the non-dialysable I^{125} radioactivity) was injected into each mouse, and the ensuing response (HA = 1:40 – 1:80) was inferior to that obtained with 72 ng native antigen (1:80 – 1:640).

(3) Those mice that developed (weak) responses (HA = 1:40) from oral priming with the native antigen (Group V) showed no enhanced clearance of the antigen.

8.4   Discussion

It is known that repeated oral feedings of BA to mice will
elicit specific antibodies in the circulation, with significant
levels of IgA (Horsfall and Rowley 1979). Using a more defined
and sensitive system we have shown that a single oral dose of BA
(as little as 0.01 mg), although incapable of eliciting antibody
production per se, could prime mice for an anamnestic, humoral
response when challenged with a booster dose 3-4 weeks later.
However, despite the presence of haemagglutinating antibodies in
their serum, these orally-primed mice did not show enhanced
clearance of the specific antigen. The possibility that this was
due to the presence of IgA antibodies whilst intellectually
appealing, could not be well substantiated.

Thus, the findings indirectly show that immunogenic BA was
absorbed from the gastrointestinal tract. While attempts to
detect it directly in tissues were unsuccessful, immunogenic
activity was however detected in the plasma of mice fed with BA.
This activity was only found early in the absorption (1/2 - 1 h),
and the absorbed BA behaved differently from the native antigen in
that the immune response induced was qualitatively different.
Unlike those primed i.v. with the native antigen, the mice primed
i.v. with the plasma extracts showed no immune elimination of the
inducing antigen despite the production of specific circulating
antibodies (like mice primed orally with the native antigen).

The same kind of "processed" antigen was also obtained from the
dialysed contents of everted ileal sacs incubated in the antigen
solution. Thus, mice primed i.v. with it did not clear the BA

although circulating antibodies were produced to it. Further, the absorbed antigen appeared to be significantly less immunogenic than the native antigen. This _in vitro_ model therefore, has tremendous potential, since large amounts of the absorbed material can be obtained for further study.

Immunogenic activity was recovered from the washed small intestine up to 2 h after antigen feeding. The type of response induced by extracts of the Peyer's patches appeared to resemble that induced by the "processed" antigen. It is however difficult to draw absolute conclusions here because the isolated mouse Peyer's patch inevitably also contains contaminating antigen adhering to adjoining tissues.

Finally, it is essential to examine the reliability of the techniques used, on which the above assumptions are based. The immune clearance test, as measured by the relative distribution of the specific I^{125} antigen in the liver and circulation (the LB index), is sensitive, accurate and objective when used for BA. Whilst it has certain drawbacks, it is nevertheless valid to use it qualitatively to gauge whether enhanced clearance is present or not. The sensitive HA assay was usually performed in duplicates or more, and the results were often confirmed by the less-subjective RIA. This latter assay is unfortunately ill-suited to detecting antibodies of low avidities (see 4.6.5), which presumably would be the kind predominantly present in the early sera used in the studies. This may be the reason for the poor detection of IgA in suspected samples (see 9.5).

8.5   Conclusions

Immunogenic BA was absorbed from the adult mouse intestine both
in vivo and in vitro. Thus, the absorbed material recovered from
the plasma or everted gut sacs primed normal mice for a systemic,
humoral response when injected i.v..   The absorption was also
shown indirectly in mice that were fed the antigen and became
primed to it systemically. However, in all the mice so-primed,
the ensuing response was qualitatively different (due to IgA
produced ?) from that of mice primed i.v.   with the native
antigen.

9.   CHAPTER 9. GENERAL DISCUSSION

The findings of this study on the absorption of macromolecules from the adult intestine are reviewed here in relation to the observations of others. In addition, the immunobiological significance of these findings will be discussed.

9.1   Are Macromolecules Absorbed from the Adult Intestine?

The general consensus of opinion is that macromolecules are not only absorbed from the gut of neonatal mammals, but also that this process continues in the adult, although at diminished rates. However, the amount of macromolecular absorption in the adult is in dispute (Lancet 1978). For instance, in the intact animal, the amount of an antigenic dose absorbed into the blood ranged from 0.00004% (Thomas and Parrott 1974) to about 5% (Hemmings and Williams 1978), while in in vitro models, the absorption ranged from 0.0016 % (Brandtzaeg and Tolo 1977) to 0.4 % (Nolan et al 1977). Our findings suggest that these discrepancies are partly due to (1) the technique (model) or (2) the antigen used, while in addition, Swarbrick et al (1979) have shown that wide variations may also be obtained among individual mice for the same antigen and system.

One of the difficulties in comparing rates of macromolecular absorption lies in defining what is absorbed (see Chapter 2). Obviously, for any antigen, the highest absorption rate is obtained by determining the amount of radiolabelled antigen absorbed in terms of the "high molecular-weight radioactivity".

However, much of this material may not be immunologically active.[*] This is shown by the lack of precipitability by specific antibodies (as with the non-dialysable H^3 DNP-FLA recovered from mouse serum) or the decreased ability to stimulate antibody production (see 9.5.2). On the other hand, the apparent lack of activity may be due, in part, to deficiencies of the detecting system e.g. the reagent antibody is not avid enough or it does not recognise new determinants generated <u>in vivo.</u> Thus, more FLA appeared to be absorbed than BA when assessed in terms of the non-dialysable radioactivity recovered in the serum, but this was not so when the absorbed antigens were measured by immunoassay (6.2).

The absorption rates using radioiodinated antigens (as obtained by Hemmings and Williams 1978) must be cautiously interpreted. Although the total radioactivity absorbed into the whole animal probably represents the maximal amount of antigen which could have been absorbed by the intestinal mucosa, this does not necessarily mean this amount is recoverable in intact form from the circulation or internal organs of the animal. Theoretically, this amount should (1) equal the loss of radioactivity from the intestinal tract (Parkins <u>et al</u> 1960), and (2) be obtained for any radiolabel used for the same antigen, assuming that intraluminal removal of these labels is uniform and low. The latter prediction was generally borne out in practice when H^3 DNP and I^{125} were used with both BA and FLA. Further degradation of the absorbed antigen occurs in the intestinal mucosa so that the amount of macromolecular material recovered in the circulation and tissues is less, as indicated by, for example, the non-dialysable H^3 DNP radioactivity at these sites. With the I^{125} label however,

[*] <i>e.g. see Tolo <u>et al</u> 1977.</i>

such an estimation is not possible since both degradation and deiodination exist in the mucosa (see 6.6). The antigen absorbed and localised in the liver, kidney and other tissues may be further degraded. This was particularly extensive in the case of FLA, so that the amount of circulating macromolecule recovered was relatively small. From these considerations, it is obviously necessary to define (1) the nature of the macromolecule absorbed; (2) the site of absorption, e.g. intestine, liver or blood; and (3) the time of absorption, when comparing the rates of absorption of different antigens. Pertinent to (3) is the fact that while the liver uptake of FLA declined rapidly between 1/2 and 2 h post feeding (presumably indicating total absorption of this antigen within 1/2 h and its rapid degradation in the liver), the uptake of BA on the other hand, increased during this period (indicating a slower absorption) (Fig. 6.4).

The total macromolecular absorption from the gastrointestinal tract of the intact animal is not only indicated by the total radioactivity recovered in the body or the loss of radioactivity from the tract, but also by estimations based on an in vivo technique using I^{125} specific antibody (6.3.1), and in vitro models (6.4). Estimations derived from the latter may be higher than those obtained in the intact animal, as was the case with BA. Possible reasons for this are, that in the in vitro systems: (1) the barrier function comprising the mucus, enzymes, mucosal integrity etc. may be affected; (2) longer periods of contact between antigen and absorbing surface are allowed (1 - 2 h), whereas the transit of bolus in the intact intestine may be rapid; and (3) the greater ease of recovery of absorbed material from the isolated intestine.

Finally, it is also apparent that different absorption rates will be obtained for different antigens, even using standardized procedures. As illustrated by the use of BA and FLA, the in vivo degradability and the physico-chemical nature of any antigen are important parameters in its absorption. The molecular size and chemical relatedness to the mucoproteins apparently govern diffusibility in the mucus (Edwards 1978) and subsequent absorption. *Molecular selectivity was also observed by Brandtzaeg & Tolo 1977.*

9.2   Where are Macromolecules Absorbed?

From everted gut sac studies, the mouse ileum appeared to absorb more BA and BSA than the jejunum. Whether this is so in vivo is unclear, but conceivably, this depends on how much intact antigen is left for absorption in the ileum after the loss in the proximal gut (due to absorption and digestion). In the case of BA, a significant amount of I^{125} antigen was found in the caecum and colon of mice 3 h post feeding. (Fig. 7.1)

The Peyer's patches have been strongly implicated in recent studies for their role in antigen-sampling and the initiation of immune responses in the gut. Thus, an accumulation of particles were found in these organelles after oral administration, e.g. horseradish peroxidase (Owen 1977), latex (LeFevre et al 1978) and carbon (Joel et al 1978). However, we were not able to demonstrate this using I^{125} or H^3 DNP-labelled BA in mice fed with this antigen 1/2 - 1 h previously. Unlike other workers who have used microscopic techniques, we estimated the antigen contained in the Peyer's patch and non-Peyer's patch tissues by

direct counting. Surprisingly, more radioactivity was found in the non-Peyer's patch tissues (Fig. 6.4 Ab). This may be due to non-specific binding of the antigen to the mucus, which is less prevalent over the Peyer's patches (Owen 1977; Page-Faulk et al 1971). However, subsequent removal of the mucus with DTT following absorption did not reveal preferential uptake of the antigen by the Peyer's patches. The problem of non-specific adherence of the antigen to the mucus may perhaps be avoided if the absorption is studied at later times after feeding, when the bolus has passaged through the intestine. However, there was no indication that BA was retained by the small intestine after 3 h (Fig. 6.4 Ab) and certainly, no immunogenic activity was detected in the Peyer's patches at 24 h following antigen feeding (Table 8.1). It remains to be seen whether chronic feeding of the antigen (as used by LeFevre et al 1978 and Joel et al 1978) would increase the uptake and retention of BA by the Peyer's patches. Although our studies did not demonstrate an accumulation of BA in these organelles, this should perhaps be attributed to experimental difficulties, and does not deny the functional importance of the Peyer's patches in the "sampling" and "processing" of antigens.

9.3   What is the Significance of Macromolecular Absorption?

The intestinal absorption of small amounts of macromolecules may be a normal process which does not result in any evident, adverse effects. For example, Jacob et al (1977) detected endotoxin in the portal blood of apparently healthy people. However, this amount may be detrimental in certain situations (e.g. atopic individuals or people with liver cirrhosis), while the absorption

of larger amounts of antigen (e.g. due to a breakdown in the barrier function of the gut) may result in endotoxaemia, etc. even in normal subjects. Using an experimental model (6.5), we demonstrated that mice immunized intraperitoneally to produce large amounts of circulating anti-BSA antibodies, rapidly died (presumably from anaphylaxis) if challenged with suitable amounts of BSA both intravenously and orally. The fact that normal animals do not suffer adverse effects from the continued ingestion of food suggests the existence of control mechanisms in preventing this. Conceivably, these mechanisms operate at 2 levels:

(1) The amount of macromolecular absorption from the gut to the circulation is limited by local intestinal factors, e.g. mucus, coproantibodies, mucosa, etc.

(2) The systemic response induced by oral immunogens may be different from that obtained by systemic stimulation, i.e. it is not "anaphylactic".

The discussion to follow will be based on these possibilities.

9.4   Natural Barriers to Macromolecular Absorption

Our findings support the concept (Edwards 1978) that the mucus is an important barrier to the absorption of macromolecules, since absorption rates were found to be inversely correlated with the presence of mucus in everted gut sacs (Table 7.2). In addition to its direct function as a physical sieve, it also contains numerous degradative enzymes as well as immunoglobulins (see below). Further degradation (with little or no retention) of the absorbed antigen in the intestinal tissues probably occurs. Any antigen that escapes these barriers is filtered by the liver and the

mesenteric lymph nodes (for some antigens e.g. BA) or the kidneys (for others e.g. FLA). The efficiency of the liver function probably varies for different antigens (see 1.6). For instance, relatively more FLA was sequestered here than BA. Thus, the ratio of non-dialysable radioactivity recovered in the whole, perfused liver to that in 1 ml serum at 1/2 h following the feeding of H^3DNP-labelled antigens to mice was 138:1 for FLA and 6:1 for BA (Section 6.2). (If the antigens were administered i.v., the liver also sequestered more FLA than BA. However, for each antigen, the liver uptake by this route was considerably less than when the antigen was given orally - this is similar to the findings of Thomas and Vaez-Zadeh (1974). Thus, the total radioactivity recovered in the whole, unperfused liver to that in 1 ml blood at 5 min following the i.v. injection of I^{125}-labelled antigens was 1.2:1 for FLA and 0.4:1 for BA (Fig. 5.3)). The rapid disappearance of FLA from the liver suggests efficient detoxification of the antigen here. This presumably is a normal process (Rippe et al 1974; Cantor & Dumont 1967) and hence stresses the importance of the liver in this regard. Consequently, the amount of absorbed macromolecule that freely circulates and becomes toxic is significantly reduced by the "natural barriers" in the body, especially for some (protein) antigens like FLA. For others like BA, this process appears less efficient, and which could perhaps account for the occurrence of endotoxaemia observed in the general population.

9.5   Immunological Barriers to Macromolecular Absorption

9.5.1   The efficiency of immunological barriers

Immunization without doubt inhibits absorption of the specific antigen. However, the efficiency of this function is disputed. This may partly depend on the sensitivity of the system used. For instance, complete blockade of BA absorption was observed in our insensitive in vivo model using I^{125} specific antibody (7.1), while no more than 58% inhibition was obtained in more sensitive systems using radiolabelled BA (7.2). On the other hand, as an exception, Swarbrick et al (1979) found significant inhibition in their highly sensitive system.

In most of these studies, the inhibition was only measured over short periods, and as such, the significance of the inhibition may be questioned. For instance, we found that the inhibition of I^{125} BA in mice was maximal at 1/2 h post feeding while this was lost at 2 h. Further, since the inhibition merely represents a reduction rather than total blockade of antigen uptake, the significance of this role is likewise questioned when large quantities of food are consumed constantly.

9.5.2   The biological significance of sIgA

The importance of the predominance of IgA antibodies at secretory surfaces (Tomasi et al 1965) remains enigmatic, since no exclusive role can be ascribed to them (see Rowley 1977). In fact, they are biologically inert compared with the other classes. Possibly, one of their major roles is to limit antigen uptake from the intestine. There is no direct evidence for this, except implied in both experimental studies (Andre et al 1974; Swarbrick

et al 1979) and clinical observations (Taylor et al 1973; Amman & Hong 1973). How well can they do this?

Although IgA antibodies can agglutinate or haemagglutinate as well as IgG or IgM, they are less easily detected using soluble antigens as in precipitation (Steele et al 1974; Klinman et al 1966; Taubman & Genco 1971). In fact, the binding curves obtained in our studies with mouse immune intestinal juice (presumably IgA antibodies; see Horsfall & Rowley 1979) in both the Farr assay and the radioimmunoassay (not shown) were disappointingly similar to those obtained with an IgA myeloma, MOPC 315 (Figs. 4.2 and 4.4). Implicitly, among other unique properties, IgA antibodies may generally have low intrinsic affinities. (The affinity of MOPC 315 for DNP is 2.0×10^6 1/M). Consequently, they may not be readily detected, especially in those systems where the affinity is limiting. In support of this, Montgomery et al (1978) found it necessary to adapt their radioimmunoassay to detect these antibodies, while Watanabe et al (1978) who observed low avidities in the IgA anti-rotavirus coproantibodies found in children, also envisaged difficulties in their detection in other systems. Although Taubman & Genco (1971) and Klinman et al (1966) observed higher affinities in IgA antibodies than the corresponding IgG antibodies, these affinities were nonetheless low (10^5 - 10^7 1/M). Furthermore, Brandtzaeg (1978) had raised the possibility that the number of specific IgA antibody-forming cells in the intestine could be underestimated for similar reasons. It is tempting to speculate further that IgA is perhaps the direct analogue of IgM in secretions. Perhaps it was specially evolved from IgM to suit the external environment (more resistant, non-anaphylactic, etc. - see below), and because of its recent

divergence (about 200 million years ago, see Nisonoff et al 1975), it is in many ways similar to IgM. Thus, like IgM (see Macario and Macario 1975), it may possess the following features:

(1) It is usually polymeric (see Nisonoff et al 1975; Tomasi & Grey 1972).

(2) Its intrinsic affinity may not increase significantly, although the functional affinity (avidity) may mature (Fiebig et al 1977; Vicari & Courtenay 1977). Indeed, Klinman et al (1966) found that while the affinity of IgG anti-LAC (hapten) antibodies in the horse progressively increased, that of IgA remained constant (at 10^7 1/M). In direct contrast however, Makela et al (1967) observed a maturation in the affinity of IgA anti-NIP antibodies in rabbits. However, the authors here admitted possible contamination of their IgA preparations with low-affinity IgM antibodies to account for the apparent low affinities of the early IgA antibodies; on the other hand, it is equally likely that the apparent increase was due to contamination with high-affinity IgG antibodies at later times. The importance of purity of preparations in describing IgA function was recently raised by Steele et al (1974).

(3) It may only have a low and transient memory (Svehag & Mandel 1964; Uhr & Finkelstein 1963; Butler et al 1978; Bloom & Rowley 1979). Thus, Bandilla et al (1969) observed a parallel in appearance between IgA and IgM in both the primary and secondary responses to haemocyanin in man, which was different from the IgG response.

How could IgA prevent antigen uptake efficiently considering its low affinity? Perhaps, it does not, for this very reason as discussed in 9.5.1, especially with soluble antigens. However, it

can bind the more particulate antigens, e.g. bacteria, red cells etc. with greater efficiency. This is because, despite its low intrinsic affinity, its polyvalency increases its binding power for the antigen (see Winkelhake 1978; Macario & Macario 1975). Thus, any functional disadvantage arising from the low intrinsic affinity is circumvented by the polyvalency of the molecule, and this suits the purpose in the external environment where the more particulate antigens predominate. The low affinity of IgM is compensated by its rapid production on stimulation (see Cunningham 1976); perhaps these two characteristics are interrelated for teleological reasons. If IgA were like IgM in this respect (there are some indications for this; see Bandilla et al 1969 and Makela et al 1967), then this would be an advantage for its role in antigen blockade. It can be further envisaged that the IgA antibodies arm the mucus and act there rather than in the lumen, as this would be more efficient and economical. Both our findings and those of Walker et al (1974a) support this. As proposed by Edwards (1978) these molecules probably form a monolayer at the interface between the mucus and overlying fluid, with their repetitive sequences of mucin-type oligosaccharides in the hinge region (where the secretory component is attached) embedded in the mucus. Soluble antigen trapped by these antibodies is consequently held here to be degraded in situ (Walker et al 1975), or released (due to the low affinity) and "deflected" down the tract. Thus, little antigen-antibody complexes may be found free in the lumen (see Table 7.6D), whereas with the more particulate antigens like bacteria, more complexes may be found here than in the intestinal wall (Rowley 1974) by virtue of their sheer weight.

It is unlikely that the immune-complexes formed in the gut are

absorbed into the circulation (see 7.4; Walker 1975; Soothill 1977). Rather, any complexes found in systemic areas are formed in situ by the reaction of free absorbed antigen with preformed antibodies. IgA-antigen complexes are presumably not readily cleared by the phagocytic system (see Chapter 8), and this may account for the high incidence of glomerulonephritis asociated with IgA deposition (Morel-Moroger et al 1972). The lack of clearance is usually attributed to the fact that IgA is not opsonic (Steele et al 1974). Whether this again is due to peculiarities of the Fc region (e.g. lack of cellular receptors for it) or the Fab region (viz. the affinity) is uncertain. It is interesting to note that the poor affinity of the molecule alone can account for this behaviour, since immune-complexes formed from low-affinity antibodies are not readily eliminated (Soothill 1977; Bradfield 1974). Conceivably, because of its low affinity, only small IgA complexes are formed (these are also the sizes commonly found in glomerulonephritis) and further, the detection of these complexes is extremely difficult (see 7.4 and Fig. 4.2).

Although antibody affinity undoubtedly influences antibody function in immunoassays, anaphylaxis, protection etc. (see Macario & Macario 1975), it is somewhat naive to attribute the biological inertness of IgA solely to its low affinity since low-affinity IgM antibodies are highly opsonic and bactericidal, and are also precipitating. Conversely, Klinman et al (1966) observed that IgG antibodies which had lower affinities that IgA, precipitated a specific hapten whereas the latter could not. Thus, other features of the antibody molecule (e.g. the Fc region and tertiary structure) also have profound influence on its

biological activity (see Winkelhake 1978; Nisonoff et al 1975).

A special feature of IgA is the lack of secondary functions, including complement-mediated (Waldman & Ganguly 1974; Tomasi 1976) or hypersensitivity reactions (Ferguson 1976). This may in fact be the reason for its emergence and predominance in areas (gut, lung etc.) which are constantly in contact with large quantities of antigens. Thus, we demonstrated that whereas mice fed with BA mixed with immune intestinal juice (specific IgA), remained healthy, other mice fed with antigen-antiserum mixtures died rapidly (7.3.1). Although the cause of death was not investigated, this was probably due to intestinal anaphylaxis, probably involving IgG1 antibodies. The latter assumption is based on the predominance of this class in mouse serum, its anaphylactic ability, and its tendency to bind to mast and epithelial cells (Winkelhake 1978; Lehrer 1977). A useful marker in this event was the increased gut permeability to unrelated antigens. This phenomenon was further demonstrated in both in vivo (Table 7.7) and in vitro (Fig. 7.2) models, using mouse hyperimmune serum. Similar effects were found by Brandtzaeg & Tolo (1977) with rabbit IgG1 in an in vitro system, where the transport of the immunising antigen (HSA) was inhibited, while at the same time, that of an unrelated antigen (transferrin) was significantly increased. Similarly, Bellamy & Nielsen (1974) observed the emigration of large numbers of neutrophils into the intestinal lumen in pigs, either passively or actively sensitized, when challenged with the specific antigen (BSA), orally. A surprising finding in our later studies was that orally immunized mice also showed increased gut permeability when fed with the specific antigen. This implies the presence of Ig classes other

than IgA in the gut. This is plausible, since Bloom & Rowley (1979) found as many IgM antibody-producing cells as IgA cells in the lamina propria of mice immunized with live V.cholerae using a schedule of oral immunization similar to that used in our studies.[*] This may however be a normal process i.e. the gut is perhaps chronically inflamed (Clancy & Bienenstock 1976). On the other hand, the direct injury to intestinal tissues by serum antibody-antigen complexes is more apparent. Although the intestinal lesion in the germ-free mice that succumbed to the toxic BA-antiserum mixtures was not examined histologically in our studies, Germuth & Pollack (1967) were able to produce granuloma in rabbits using immune-complexes. Furthermore, IgG, IgM and complement are often present in the intestines of patients with Crohn's disease (Monteiro et al 1970; Green & Fox 1975) and coeliac disease (Booth et al 1977). In short, our findings are consistent with the contention (Brandtzaeg & Baklien 1976) that the underlying mechanisms of many of the intestinal diseases are probably immunological. In furtherance of this view, it is assumed that a major function of IgA at these sites is to modulate the phlogistic reactions. (Here again, its low affinity may aid this function since, in an analogous situation, Taylor et al (1979) found low-avidity antibodies as a cause of the prozone phenomenon). Although such a blocking role has been demonstrated in other areas (Turk et al 1970; Griffiss 1975), we were not able to demonstrate convincingly this anti-inflammatory effect at the mucosal level. There were obvious technical difficulties to account for this. For instance, too much antigen was probably used (in order to produce a measurable effect in the rather insensitive in vivo system); this may be the reason why orally immunized mice also showed increased gut permeability when fed

[*] see also Baklien et al 1972

with the specific antigen, despite the presumed presence of IgA antibodies. Nonetheless, the efficiency of this moderating function must be weighed against the fact that IgA -deficient people (occurring at a rather high frequency of 1 : 500) are apparently normal, although there is some correlation between this deficiency and increased incidences of intestinal diseases (Horowitz & Hong 1975; Cunningham-Rundles et al 1978). It is undoubtedly useful to examine the importance of this immunoglobulin in IgA-deficient animals. Unfortunately, we were not able to detect serum IgA deficiency in about 400 normal LAC mice screened (using the Mancini technique), or in those treated orally with phenytoin (Aarli & Tonder 1975), in order to obtain a useable model.

9.6   The Systemic Response to Ingested Antigens

It was seen in 6.5 that fatal anaphylaxis was produced in mice that were immunized systemically and then challenged with the immunising antigen orally. It was also discussed in the foregoing sections that this situation is rather artificial. For instance, if an antigen is normally and constantly ingested, there exists mechanisms to limit toxic amounts reaching systemic tissues. Another way in which this deleterious effect is prevented is the development of a unique response to orally-ingested antigens, as distinct from that obtained by systemic immunization (where "anaphylactic" antibodies were produced). Thus, the response could be abolished or reduced (tolerance), or the antibodies produced are not "anaphylactic". In the latter case, these antibodies are probably of the IgA type and it seems likely that these would have some modulating effect on possible adverse

reactions (see Griffiss 1975). These mechanisms are elaborated
below.

9.6.1 Sytemic tolerance to ingested antigens

Systemic tolerance was induced in rats, mice, guinea-pigs, etc.
to a variety of antigens when these were given orally (see 1.6).
This was generally indicated by a decrease in the humoral response
when the animals were tested with the inducing antigen given
intravenously. Most of the antigens studied were proteins.
Whether other types of antigen have similar tolerizing potential
is uncertain. Certainly, it appears that BA is less effective in
doing this (see Table 8.1). The mechanisms of orally-induced
tolerance remain speculative (see 1.6) but an interesting feature
(in the cases studied) is the inability to produce this effect by
i.v. injections of the same antigen over a wide dose range
(Hanson et al 1977). It thus seems that the mechanism involved is
an active one, e.g. due to suppressor T cells in the Peyer's
patches (Ngan & Kind 1978), IgA-complexes (Andre et al 1976) or
other active principles. The relevance of our findings to this
phenomenon that the absorbed BA recovered from the serum or
everted gut sacs was less immunogenic than the native antigen
(8.3.2 & 8.3.3) remains to be elucidated. In this regard,
Rothberg et al (1978) had also circumstantially inferred that the
antigenic BSA found in the circulation of rabbits fed with 0.6%
BSA was probably non-immunogenic.

9.6.2   Systemic humoral response to ingested antigens

The circulating antibodies produced in response to oral

immunization may be derived from 3 sources:

(1) Solely from gut-associated tissues.

(2) Solely from systemic tissues.

(3) From both (1) and (2).

Support for (1) comes from the rather well established concept of cellular traffic, involving the migration of sensitized cells in the Peyer's patches to the mesenteric lymph node and then back to the lamina propria. Thus, following oral stimulation, specific antibody-producing cells (viz. IgA type) were found in the lamina propria and mesenteric lymph nodes, but were absent in the spleen (Heatley et al 1977; Goldberg et al 1971), and the predominant class of circulating antibodies produced was IgA (Heatley et al 1977; Andre et al 1973). Further, Rothberg et al (1969) found systemic antibody-responses in rabbits fed with 0.1% BSA even though no immunogenic levels of circulating BSA were found in these animals, nor were specific antibody-forming cells found in the spleen or lymph node. However, in these studies, the predominant class found in the circulation was not IgA but IgG/IgM. The generation of these classes is generally attributed to the stimulation of systemic tissues by absorbed antigen, especially when antibody-forming cells of these classes are found in the spleen. On the other hand, Rothberg et al (1978) contended that the lamina propria could also be involved, since it also possessed cells belonging to these classes. There is no doubt that for most antigens, both sites may be concerned with the elicitation of the systemic response, particularly when the dose given is high. Their relative contributions presumably depend on the nature of the antigen. It is tempting to generalize that while protein antigens (HSA, ovalbumin, etc.) can tolerize an

animal rather easily when given orally (e.g. Hanson et al 1977; Sewell et al 1979), and the circulating antibodies formed are not predominantly IgA (e.g. Rothberg et al 1967; Dolezel & Bienenstock 1971), the more particulate antigens such as ferritin, RBC and BA are perhaps less tolerizing and also generate an IgA-based response (Crabbe et al 1969; Bazin et al 1970; Felsenfeld & Greer 1968).

In our studies with BA, it was found that this antigen did not induce the formation of circulating antibodies when given once orally (0.01 - 1.0 mg). However, the mice so treated were primed to the antigen, as shown by the anamnestic response that resulted from an i.v. challenge (8.1). This is similar to the findings of Rothberg et al (1973), in which rabbits fed with 0.1% BSA and then boosted i.v., were found to produce a rapid and significant increase of circulating antibodies and antibody-forming cells, even though the chronic ingestion by itself did not produce any detectable antibody-forming cell in the circulation. Control rabbits merely given the i.v. booster did not produce a response. However, unlike their findings, the ensuing response in our studies in the orally-primed mice was different from that of mice primed i.v. with the native antigen. An IgA type of response was suggested in this group, as indicated partly by the lack of immune clearance for the antigen (8.1) (although cellular studies like those of Rothberg et al (1973) would have been more informative). Similar findings were obtained when mice were primed i.v. with the absorbed antigen recovered from mouse plasma or everted gut sacs (8.3.2 & 8.3.3). In other words, the absorbed antigen was "processed" to stimulate a defined type of response (IgA?). The idea of such an "IgA-genic" antigen was in fact proposed by

Heremans in 1969 to account for the systemic IgA response to ingested antigens. However, this has not received much attention (see Dolezel & Bienenstock 1971). Any processing of the antigen must occur in the intestine, since "processed" BA was also found in everted gut sacs.[*]

In summary, the induction of systemic tolerance or a unique (non-anaphylactic) antibody response to ingested antigens may be a normal control mechanism in preventing any adverse effect which could result from macromolecular absorption.

[*]*Undoubtedly, characterisation of the "processed" antigen will be important in future studies.*

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