PHARMACEUTICAL FORMULATIONS

AS IMMUNOLOGICAL ADJUVANTS.

by

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If this is a man.

You who live safe

In your warm houses

You who find, returning in the evening

Hot food and friendly faces:

    Consider if this is a man,

    Who works in the mud

    Who does not know peace

    Who fights for a scrap of bread

    Who dies because of a yes or no.

    Consider if this is a woman,

    Without hair and without name

    With no more strength to remember,

    Her eyes empty and her womb cold

    Like a frog in winter.

Meditate that this came about:

I commend these words to you.

Carve them in your hearts

At home, in the street,

Going to bed, rising;

    Or may your house fall apart,

    May illness impede you,

    May your children turn their faces from you.

Primo Levi 1919-1987.

ABSTRACT

The aim of this work was to enhance the immune responses to ovalbumin (OVA) following its oral administration, by the association of the protein with colloidal carriers, which may protect the protein from degradation in the gastrointestinal tract and/or facilitate its uptake across the intestine.

An enzyme linked immunosorbent assay (ELISA) was established for the determination of rat anti-OVA antibodies and an immunisation protocol was established to induce a statistically significant salivary antibody response to OVA in the rat. A radioimmunoassay for the determination of rat anti-OVA antibodies was also established, to confirm the ELISA results. Methods were established to determine the extent of incorporation or adsorption of OVA into or onto the colloidal carrier formulations.

OVA was incorporated into liposomes and polyacrylamide microparticles, and adsorbed to poly 2-butylcyanoacrylate particles, and gastrically intubated into separate groups of experimental rats. The primary and memory immune responses, both sera and saliva, were compared for each formulation with suitable control and blank groups.

All colloidal carriers induced enhanced immune responses to OVA following oral administration in the

rat, when compared with the respective control group responses. However, the enhancement for the liposomal group was not statistically significant when assessed in an Unpaired Student 't' test.

The effect of particle size on the immune responses was assessed by the oral administration of 100nm and 3μm poly 2-butylcyanoacrylate particles with adsorbed OVA.

An electron microscopy study was undertaken with gold labelled poly 2-butylcyanoacrylate particles in an attempt to demonstrate the uptake of particles by M-cells overlying the Peyers' patches in the rat intestine.

CHAPTERS.

TABLES.

1.1. Approaches used in attempts to enhance the absorption of macromolecules across the gut.

1.2. Materials known to be transported by M-cells.

2.1. ELISA sera response following intraperitoneal injection of myoglobin (mean ±SD n=6).

2.2. ELISA sera response following intraperitoneal injection of ovalbumin (mean ±SD n=5).

2.3. ELISA sera response following intraperitoneal injection of ovalbumin, repeated assay of pooled sample collected day 35 (mean ±SD n=6).

2.4. ELISA sera response following intraperitoneal injection of ovalbumin, repeated assay of sample on four separate days (mean ±SD).

2.5. Counts per minute for different fractions of Iodine-125 labelled Staphylococcus aureus Protein A.

2.6. Counts per minute for different fractions of Iodine-125 labelled swine anti-sheep IgG.

2.7. Radioimmunoassay results for Iodine-125 labelled Staphylococcus aureus Protein A, counts per minute (mean n=3).

2.8. Radioimmunoassay results for Iodine-125 labelled swine anti-sheep IgG, counts per minute (mean n=3).

2.9. Lowry assay reproducibility (mean ±SD n=6).

3.1. ELISA saliva response following gastric intubation, followed by repeated salivary gland vicinity injection of BGG/DNP (mean ±SD n=6).

3.2. ELISA salivary response following repeated gastric intubation of myoglobin (mean ±SD n=6).

3.3. ELISA salivary response following repeated gastric intubation of ovalbumin (mean ±SD n=6).

4.1. Ovalbumin entrapment in liposomes as determined by Lowry assay (mean ±SD n=4).

4.2. Size analysis of liposomes by laser diffractometry.

4.3. ELISA saliva and sera responses to gastric intubation of ovalbumin in liposomes (mean ±SD n=8).

5.1. ELISA results for PA1 and PA2 sera samples (mean ±SD n=8).

5.2. ELISA results for PA1 and PA2 saliva samples (mean ±SD n=8).

5.3. ELISA results for PA1 saliva assayed with pre-inoculation saliva (mean ±SD n=8).

FIGURES

1.1. Schematic diagram of the selective and non-selective mechanisms for the absorption of macromolecules in the immature gut.

1.2. Schematic diagram of the non-selective mechanisms for the absorption of macromolecules in the mature gut.

1.3. Schematic diagram of an intestinal lymphoid follicle.

1.4. Schematic diagram illustrating the process by which antigens are absorbed via M-cells.

1.5. Flow diagram of the sequence of events involved in the immune response to intestinally absorbed antigen.

2.1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of (1) native ovalbumin, (2) ovalbumin incorporated in liposomes and ovalbumin adsorbed to (3) beta-cyclodextrin and (4) dextran 70 stabilised poly 2-butylcyanoacrylate particles.

2.2. Developed immunoblot of ovalbumin samples (1) - (4) described in legend for Figure 2.1. after their electrotransfer to a nitrocellulose membrane.

2.3. Phenylisothiocyanate derivatization of amino acids to produce phenylthiocarbamyl amino acids for analysis by high performance liquid chromatography.

3.1. A simplified flow diagram illustrating the production of systemic tolerance at the same time as local antibody production.

5.1. Mean particle size of polyacrylamide microparticles determined by Coulter counter (n=4).

6.1. Diagramatic representation of photon correlation spectroscopy apparatus.

6.2. Diagramatic representation of laser diffractometry apparatus.

6.3. Particle size analysis of beta-cyclodextrin stabilised poly 2-butylcyanoacrylate particles by Coulter counter.

7.1. Electron micrograph of putative M-cell in the epithelium overlying the Peyer's patch in a Wistar rat. x 6,485.

7.2. Electron micrograph of putative M-cell in the epithelium overlying the Peyer's patch in a Wistar rat. x 11,923.

ABBREVIATIONS

ABTS   -   2,2'-azino-di-(3-ethylbenzthiazoline sulphonate)

Az   -   Azide

BDMA   -   Benzyldimethylamine

BGG   -   Bovine gamma globulin

BSA   -   Bovine serum albumin

CHOL   -   Cholesterol

c.p.m. -   Counts per minute

DDSA   -   Dodecyl succinyl anhydride

DNP   -   2,4-Dinitrophenol

DSPC   -   Distearoylphosphatidylcholine

EDTA   -   Ethylenediaminetetraacetic acid

ELISA   -   Enzyme linked immunosorbent assay

FCA   -   Freunds' complete adjuvant

GALT   -   Gut associated lymphoid tissue

g.i.   -   Gastric intubation

g.i.t. -   Gastrointestinal tract

HPLC   -   High performance liquid chromatography

HRP   -   Horseradish peroxidase

IEL   -   Intraepithelial lymphocytes

IF   -   Intrinsic factor

i.p.   -   Intraperitoneal

MYO   -   Horse heart myoglobin

NC   -   Nitrocellulose

OVA   -   Ovalbumin

PA   -   Polyacrylamide microparticles

PAGE   -   Polyacrylamide gel electrophoresis

p(BCA) -   Poly 2-butylcyanoacrylate

PBS   -   Phosphate buffered saline pH 7.4

PCS   -   Photon correlation spectroscopy

p.i.   -   Pre-inoculation

PP   -   Peyers' patches

RIA   -   Radioimmunoassay

rpm   -   Revolutions per minute

SDS   -   Sodium dodecyl sulphate

SGV   -   Salivary gland vicinity

TEMED   -   N,N,N',N'-tetramethylethylenediamine

TRIS   -   Tris(hydroxymethyl)aminomethane

Tsi   -   T suppressor inducer cell

Tsup   -   T suppressor cell

INTRODUCTION

Progress in the field of biotechnology has renewed interest in the oral delivery of macromolecules. However, whilst small quantities of proteins and other macromolecules may be absorbed from the gastrointestinal tract intact (1-5), enzymatic degradation and poor absorption have limited the delivery of therapeutically active molecules such as insulin and heparin. Several mechanisms, both specific and non-specific, exist in the gut by which these materials pass from the lumenal surface to the systemic circulation. Future developments in the oral delivery of proteins is likely to depend on the exploitation of these normal physiological processes. However, the uptake of macromolecules is normally associated with lysosomal degradation which markedly reduces the amount of intact macromolecule reaching the circulation.

Uptake of macromolecules by M-cells, which are specialised cells overlying the lymphoid follicles of the gastrointestinal tract and are involved in antigen uptake, does not appear to involve extensive degradation. However, the primary function of the M-cell is to provide the immune system with samples of gut antigen in order to stimulate the body's defence mechanisms against future absorption of the antigen.

Although the response of the immune system to absorbed antigen will limit the oral delivery of proteins, this may provide an attractive alternative to parenteral immunisation. The oral administration of antigens may prove to be a highly efficient method of producing secretory IgA at all mucosal sites and may be of great benefit in immunisation protocols which call for IgA antibody production.

This project is concerned with investigating the immune responses, both serum and secretory, to a model protein administered orally. The protein was incorporated into, or adsorbed onto, colloidal carriers which were designed to confer protection of the protein against enzymatic destruction in the gastrointestinal tract (g.i.t.) and/or to facilitate uptake of the protein by M-cells. The overall aim was to enhance the immune responses induced by the protein, particularly the secretory immune response. If successful, this research effort may lead to applications in the development of effective oral vaccines against a number of organisms.

1.1. Evidence of intestinal absorption of proteins and macromolecules.

During the perinatal period, proteins and macromolecules can readily penetrate the immature gastrointestinal mucosa. It had been assumed that this process ceases with maturation of the epithelial cells. However, increasing clinical and experimental evidence has shown that small amounts of antigenically active proteins and macromolecules can penetrate the adult intestinal epithelium (1-5).

The clinical consequences of the exposure of the intestine to proteins are either the induction of an immune response or the loss of systemic reactivity to parenterally administered antigen (systemic tolerance). As early as 1935, Wilson and Walzer (6) demonstrated the presence of serum antibodies to proteins ingested in physiological quantities. This suggests that undigested protein can be absorbed and distributed throughout the body. Similarly 15-30% of normal adults have been shown to develop milk antibodies after ingestion of a small amount of milk proteins (7). An increase in the permeability of the gut to macromolecules is associated with certain intestinal disease states, and it is possible that the associated

rise in antigen absorption may account for some of the extra-intestinal symptoms of these conditions (8,9).

Experimental investigations have clearly demonstrated that proteins and macromolecules can be absorbed intact from the gut. Warshaw et al (1) infused the enzyme horse radish peroxidase (HRP) through an indwelling catheter into the jejunum of rats and found that small amounts of protein tracer were consistently transmitted across the intestinal mucosa. Maximal portal blood concentrations ranged from 17.2 to 113.4 ng/ml after administration of a dose of 23mg/kg, and a maximum of 0.153% of the administered dose was absorbed. Cornel et al. (10) reported the absorption of similar concentrations of HRP after intralumenal injection into ligated segments of jejenum and ileum in rats. Histological examination of the tissue showed HRP adsorbed to the apical surface membranes, within membrane bound cytoplasmic canalicular, vesicular and vacuolar structures, in the intercellular spaces between absorptive cells, traversing the basement membrane and within the spaces of the lamina propria.

Comparison of the absorption of bovine serum albumin (BSA), HRP, elastase and chymotrypsin following infusion into rat duodenum, showed that 100 to 1000 times more BSA than HRP was absorbed and that elastase and chymotrypsin were absorbed in quantities

intermediate between BSA and HRP (3). The reasons why there are such differences in the absorption of various proteins is not known, but it is likely that absorption depends on characteristics such as molecular weight, charge, configuration and structure. Bernstein and Ovary (11) found that small molecular weight bivalent haptens frequently provoked passive cutaneous anaphylaxis reactions in experimental animals, whereas larger molecular weight haptens had no effect. They concluded that molecular size is an important factor controlling the absorption of a given protein from the gut.

In 1959 Danforth and Moore demonstrated the production of hypoglycaemia in rats following injection of insulin into ligated intestinal loops (12). There have been many attempts since, to increase the intestinal absorption of insulin and other proteins and macromolecules using a variety of formulations. Only limited success has been achieved and much of the data generated are confusing and contradictory.

In early efforts to promote absorption, proteins were coadministered with agents which prevented proteolysis such as Aprotinin (trasylol) (13) and pancreatic trypsin inhibitors (12). Whilst there was some increase in absorption, for a significant effect to be achieved large quantities of protein had to be administered.

Enzyme Inhibitors	pancreatic trypsin Inhibitors	12
	trasylol	13
Absorption enhancers	ionic surfactants	14, 15
	non-ionic surfactants	16, 17
	salicylates	18
Lipid carrier systems	liposomes	19-26
	mixed bile salt micelles	29, 30
	oil-in-water emulsions	31, 32
	water-in-oil-in-water emulsions	33-38
Particulate carrier systems	nanoparticles	38, 39

Table 1.1. Approaches used in attempts to enhance the absorption of macromolecules across the gut.

Alternative approaches that have been investigated include the use of absorption enhancers, lipid carrier systems and particulate carriers (Table 1.1.). However, these studies did not really deal with the possible immunological consequences of enhanced macromolecular absorption.

1.2. Attempts to enhance absorption.

1.2.1. Absorption enhancers

Some early workers used ionic surfactants to enhance the absorption of insulin, but these agents were found to irritate the gastric mucosa (14). More success was achieved with non-ionic surfactants eg. Brij (15,16) and Cetomacrogol (17), but high concentrations of insulin were required.

Sodium-5-methoxysalicylate has been shown to enhance insulin absorption after intraduodenal administration to rats. But high concentrations of salicylate (>6mg/rat) are required at the absorption site, so a rectal formulation, where dilution effects are reduced, is more suitable (18).

1.2.2. Lipid carrier systems

1.2.2.1. Liposomes

Liposomal systems have been used by several groups (19-23) in attempts to increase the absorption of macromolecules. Much of this work has been associated with the attempted development of an orally effective formulation of insulin.

In a recent comprehensive review on liposomes, Woodley (24) concluded that they are not suitable for the delivery of insulin and other macromolecules in humans, as only limited and variable absorption has been achieved with this type of formulation. However evidence exists which indicates that liposomes can act as immunological adjuvants, potentiating the immune response to orally co-administered antigens (25-28). This is discussed further in chapter 4.

1.2.2.2. Mixed micelles

The duodenal absorption of heparin in rats was enhanced when administered in a mixed micellar solution, probably due to enhanced fluidity and permeability of the intestinal wall (29). However, these systems were much more effective in the large intestine, due to the reduction of dilution effects at this site (30).

1.2.2.3. Emulsions

The enhanced intestinal absorption of heparin, in oil-in-water emulsions (31,32) in rats and gerbils, and of insulin, in water-in-oil-in-water double emulsions (33-37) in rats has been reported. These lipid formulations were thought to promote absorption by protecting the proteins from degradation, but the possible effect of the surfactants on the intestinal membrane was not considered.

1.2.3.Particulate carriers

Couvreur et al. (38) orally administered to rats, insulin adsorbed onto a range of hydrolysable polyalkylcyanoacrylate nanoparticles. The nanoparticles were spherical with a mean diameter of 200nm and a highly porous structure. No hypoglycaemic effect was observed. Oppenheim et al. (39) achieved more success by using nanoparticles (200nm) of insulin produced by desolvation and cross linking with glutaraldehyde of neutral insulin injection. Following intrajejunal administration, a hypoglycaemic response was detected in both rats and mice but the site and mechanism of absorption were unknown.

Although many investigations have clearly demonstrated that intact proteins and macromolecules can be absorbed across the gastrointestinal mucosa, the

quantity that is absorbed is small. Despite

considerable efforts to promote uptake by the use of

suitable formulations, the extent of absorption that

can be achieved is still insufficient to produce

therapeutic plasma levels of compounds such as insulin

following oral administration.

1.3. Mechanisms of protein and macromolecular absorption.

1.3.1. Macromolecular absorption in the immature intestine.

The permeability of the gastrointestinal mucosa to

proteins during the perinatal period seems to be

related to a number of factors. These include decreased

breakdown of macromolecules, increased attachment of

molecules to the intestinal epithelial surface and less

inhibition of uptake by immunoglobulins in the milk and

colostrum. Within the same animal the mechanism and

capacity for macromolecular uptake by the enterocytes

may change from one region of the gut to another. In

addition there are species related differences in

permeability. The rat and the mouse exhibit selective

absorption, involving a pH dependent IgG (Fc) receptor

in the proximal small intestine, during the first two

and three weeks of life respectively (40). Both show

rapid proteolysis from birth and no proteinuria. In contrast, in ungulates and carnivores, absorption is non selective, almost every colostral protein is absorbed, proteolysis is suppressed by colostral trypsin inhibitor and there is physiological proteinuria. This lasts only for the first two to three days of life, although the enterocytes will internalise macromolecules for longer periods they will not transport them.

There appear to be two mechanisms possible for the absorption of proteins through the neonatal intestine, either receptor or non-receptor mediated transport (Figure 1.1.). The receptor mediated transport involves the formation of coated pits and vesicles (41,42). It is thought that specific protein receptors are present in the walls of the microvilli and that a substance called clathrin floats freely within the structure of the microvilli. When a macromolecule binds to its specific receptor, the clathrin binds to the other side of the same receptor and a complex is formed. This moves to the base of the microvillus where a clathrin coated pit is formed which then breaks free to form a distinct vesicle. The vesicles traverse the cell and undergo exocytosis to release the macromolecule into the extracellular space (43,44). The clathrin coat apparently protects the vesicle from fusing with the
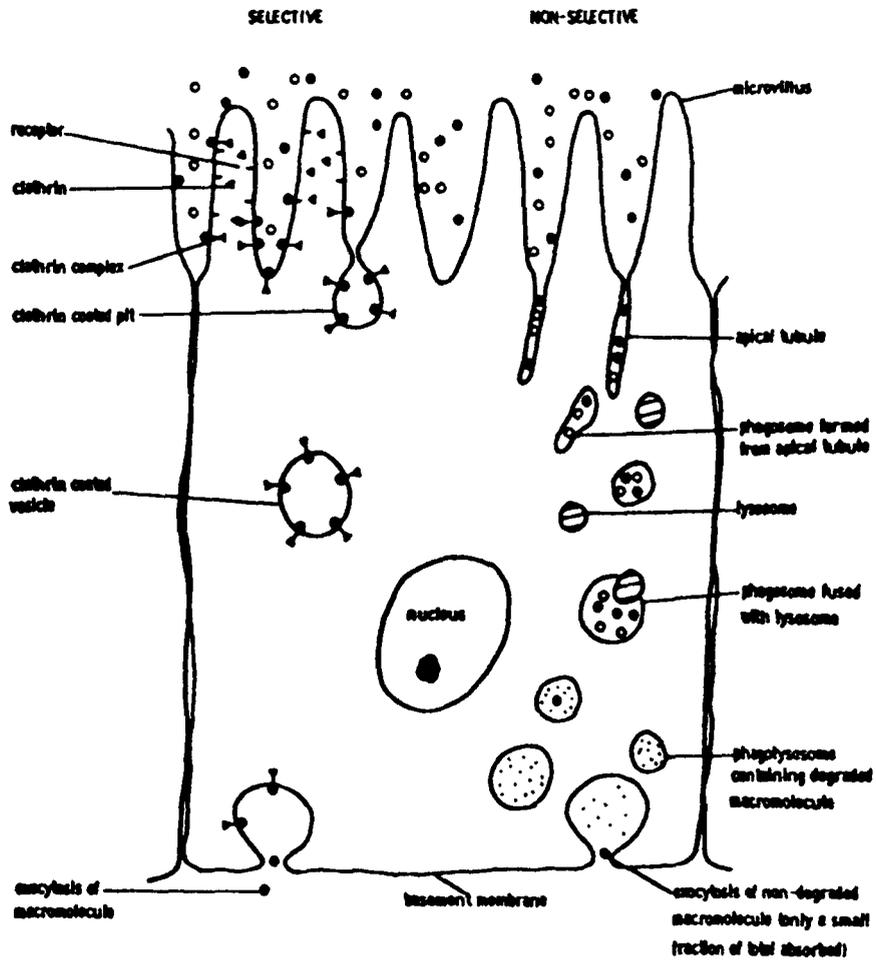
Figure 1.1. Schematic diagram of the selective and non-selective mechanisms for the absorption of macromolecules in the immature gut.

lysosome. The cells in the upper areas of the intestine display receptor mediated transport whilst the cells lower down in the small intestine do not. Non-receptor mediated transport is thought to occur via non-selective apical tubular systems in the cell surface. The end of these tubules pinches off to form vesicles which pass through the cell and are exocytosed (43,44). Only a small proportion of the macromolecules absorbed by the non-receptor mediated transport mechanism actually reaches the systemic circulation, the rest undergoes digestion by lysosomal enzymes after fusion with the cell lysosomes.

The extensive uptake of macromolecules ceases with the development of the intestinal immunological and non-immunological host defences and maturation of the intestinal epithelial cells. As the cells mature they no longer internalise macromolecules, a process known as closure. In humans closure is more subtle than in other animals and the major changes in absorption properties of the intestine are thought to occur in utero.

1.3.2. Macromolecular absorption in the mature intestine.

Following closure the uptake and transmission of macromolecules does not cease completely, several

mechanisms exist by which such compounds can still penetrate the intestinal mucosa.

Combined morphological and physiological experiments have shown that the mature small intestinal epithelial cell is able to engulf macromolecules by an endocytic process indistinguishable from the pinocytosis described for human macrophages (Figure 1.2.). The initial event in this process is the adsorption of large molecules onto components of the microvillus membrane of intestinal absorptive cells. When a sufficient concentration of molecules is in contact with the cell membrane invagination occurs and small membrane bound vesicles (phagosomes) are formed. The macromolecules then migrate within the phagosomes to the supranuclear region of the cell where they coalesce with lysosomes to form large vacuoles called phagolysosomes. Within these structures intracellular digestion occurs but small quantities of ingested molecules are thought to escape degradation. The phagolysosomes migrate to the basal surface of the cell and release their contents including any non-degraded macromolecules, into the interstitial space by a reversal of the pinocytotic process. In vitro studies on the intestinal absorption of tritiated horseradish peroxidase in adult rabbit jejunum suggested the existence of at least two functional pathways for

INTRA-CELLULAR PATHWAY   INTER-CELLULAR PATHWAY ?

microvillus

phagosome produced
by endocytosis

nucleus

tight junctions

lysosome

phagosome fused with
lysosome to form a
phagolysosome

phagolysosome containing
degraded macromolecule

basement membrane

exocytosis of non-degraded
macromolecule (only a small
fraction of total absorbed)
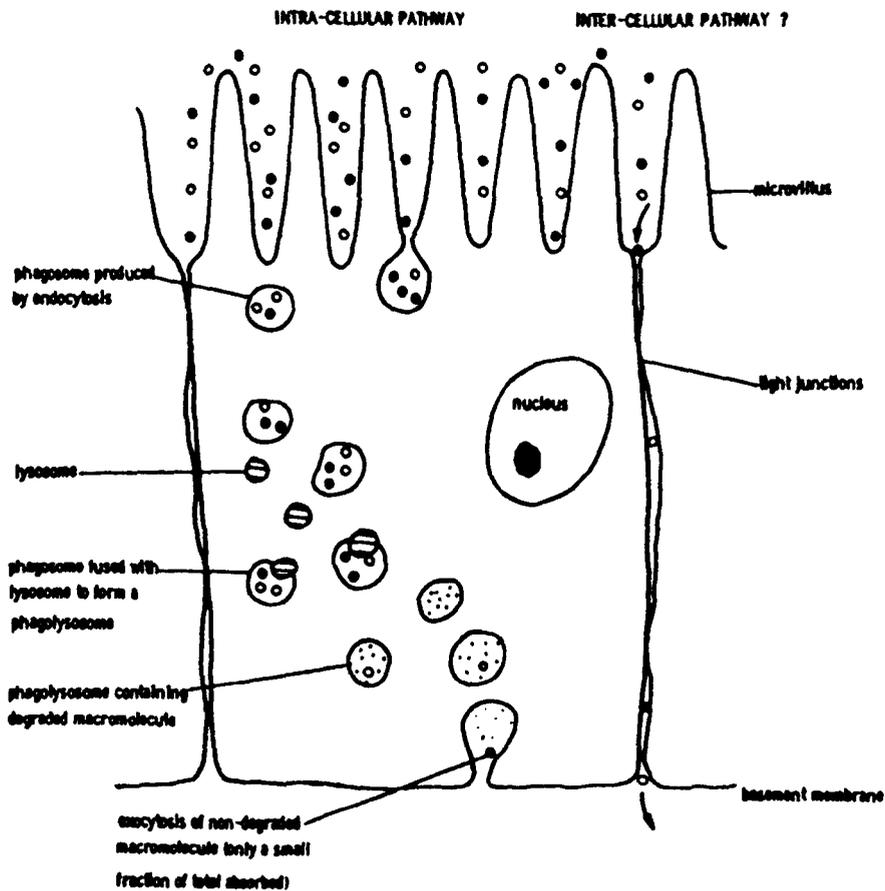
Figure 1.2. Schematic diagram of the non-selective mechanisms for the absorption of macromolecules in the mature gut.

intestinal protein transport (45). The main route
involves endocytosis with striking intracellular
degradation as described above. It was estimated that
67-97% of absorbed HRP was degraded whilst crossing the
tissue from the mucosa to serosa. A second direct
pathway exists which allowed HRP to cross the mucosal
barrier intact but that required the structural and
metabolic integrity of the epithelial cell. The
location of this direct route is not known and may be
either intracellular or intercellular through tight
junctions.

Further work with HRP demonstrated that after
infusion into the rat jejunum through an indwelling
catheter, small but significant amounts of protein were
consistently transmitted across the gut and penetrated
into lymph and portal blood (1). A number of studies
with compounds other than HRP has also shown that the
lymphatic system can contribute significantly to the
transport of absorbed macromolecules into the systemic
circulation. As early as 1936 Alexander et al. (46)
found that egg white protein, absorbed intact from the
intestinal tract of dogs, reached the vascular
compartment via the lymphatic vessels. May and Whaler
(47) using Clostridium botulinum toxin A (molecular
weight 900,000) showed that in rats, rabbits and mice
higher titres of toxin were often present in the

thoracic duct lymph than the peripheral blood,

indicating that absorption was occuring via the

lymphatic system. A similar conclusion was reached by

Gans and Matsumoto (48) after investigating the

absorption of <u>Escherichia coli</u> endotoxin in rats.

Not all macromolecules and proteins that are absorbed

from the intestinal lumen pass directly into the

lymphatic system. The macromolecule ferritin is taken

up by the intestinal absorptive cells and has been

found, both as aggregates and as individual particles,

in macrophages, fibrocytes and endothelial cells of the

lamina propria (49). Investigations into the absorption

pathway of heparin and insulin have shown that a

greater percentage of both molecules is absorbed into

the portal vein (32,50). The proportion of the absorbed

dose which passes into the lymphatic vessels can vary

considerably. In a series of experiments Katayama and

Fujata (51,52) examined the intestinal absorption of

three enzymatic proteins, $1'2'-(^3H)$-Coenzyme Q10

$(^3H$-Q10), ^{131}I labelled elastase (molecular weight

24,000), and ^{131}I-lysozyme. The absorption of each

molecule was low, only 0.05-2.06% of the total dose,

but the importance of the lymphatic vessels in the

absorption process varied considerably. Eighty per cent

of the absorbed ^3H-Q10 was recovered from the lymph,

predominantly in the chylomicron fraction (lipoprotein

units), whereas the portal vein was the main absorption
pathway for ^{131}I-lysozyme. During the first 7 hours
after administration of ^{131}I-elastase the lymph levels
were 10 times higher than the plasma levels and 36% of
the absorbed dose was calculated to have been absorbed
via the lymphatic system. The authors concluded that
absorption into the lymphatic vessels was dependent on
the physico-chemical properties of the macromolecules.
They suggested that further work was required to
determine the relationship between these
characteristics of macromolecules and uptake into the
lymphatic system.

Within the intestinal region blood flow is
approximately 500 times that of lymph flow. This means
that a larger percentage of absorbed material will move
into the portal vein unless there is a selective
mechanism for uptake into the lymphatic vessels. Some
understanding of the factors controlling absorption
into the lymphatic vessels can be obtained by
considering the physiological functions of this system.
Its primary function is the collection of tissue fluid
from extracellular spaces, which is then returned to
the vascular compartment via the lymphatic system.
Plasma proteins in the tissue fluid which are too large
to permeate through the capillary pores back into the
blood vessels are returned to the systemic circulation

by the same route. The lymphatic vessels are therefore structures adapted to the collection and transportation of large molecules. It is likely that absorbed macromolecules which are too large to permeate the intestinal blood capillaries will be absorbed into the mesenteric lymph duct. In the small intestinal region the lymphatic vessels also have a specialised function, that of fat absorption. Lipid digestion products that are too lipophilic to readily disperse in the aqueous environment of the blood are packaged into lipoprotein units (very low density lipoproteins and chylomicrons) which pass into the lymph. Lipophilic nutrients such as cholesterol and the fat soluble vitamins associate with the chylomicrons within the mucosal cell and are therefore transported via the lymphatic system. It has been shown that highly lipophilic exogenous compounds can also be selectively absorbed into the lymphatic vessels in the same way. The presence of the coenzyme ^{3}H-Q10 in the chylomicron fraction of the lymph suggests that this molecule is highly lipophilic and therefore associated with the chylomicrons within the mucosal cell and was selectively absorbed into the lymphatic vessels. Thus the uptake of macromolecules into the lymphatic vessels will be dependent on a number of physico-chemical properties including lipophilicity, molecular size and molecular

configuration, as was concluded by Katayama and Fujata (51,52). Macromolecules in the lymph will rapidly come into contact with the lymphocytes in the draining lymph nodes which are the inducers of immune responses.

Highly specialised mechanisms also exist along the intestinal tract for the absorption of specific macromolecules. Vitamin B12 cobalamin is a dietary coenzyme required to sustain life in all mammalian species. It is transported across the ileal enterocytes to the portal circulation by a process involving a specific ileal mucosal receptor. In the gut vitamin B12 binds to a specific gastric glycoprotein called intrinsic factor (IF), which has an estimated molecular weight of 50,000-70,000 and is secreted by the parietal cells (53). The binding of vitamin B12 and IF is rapid and the complex formed is very stable over a wide range of pH conditions and in the presence of gut enzymes (54). When the vitamin B12-IF complex reaches the ileum, it in turn binds specifically to a receptor present on the surface of the enterocytes. The attachment of the complex to its receptor is calcium dependent, does not require metabolic energy and occurs preferentially between pH 6.0 and 8.0 (55). The actual uptake of the complex by the ileal enterocyte is poorly understood. It is a slow (>3hr), energy dependent process (56), and seems likely to involve some sort of

adsorptive endocytic mechanism. It is possible that similar mechanisms exist for the absorption of other specific macromolecules which as yet are unknown.

The uptake of some plant and bacterial protein toxins is initiated by binding to the surface of the enterocytes. Many of these toxins consist of two subunits, with subunit A being a lectin which facilitates the entry of the toxic B subunit into the cell (40). Several viruses eg. influenza virus, and bacteria eg. E.Coli, anchor themselves on the cell with lectins before penetration.

Another specialised mechanism exists within the gastrointestinal tract by which small quantities of macromolecules (antigens) may be absorbed. Absorption occurs via the M-cells which are associated with the lymphoid tissue of the gut and can result in the generation of an immune response. This process is discussed in detail in section 1.5.

1.4. Intestinal absorption of particles.

Since 1850 a number of researchers have reported the passage of particulate material across the adult small intestine. A series of studies by Volkheimer (57-62) indicated that diatoms, pollens, spores, cellulose particles, plant cells and starch grains can cross the intestinal mucosa in man by a process called

persorption. Many more particulates have been shown to cross the intestinal mucosa of animals and particulates of intestinal origin have been reportedly found in urine and cerebrospinal fluid (60), and in the cordblood of newborn human infants after ingestion of the particulates by the mothers (62).

Volkheimer described persorption as a mechanical process whereby "large", solid particles are 'kneaded' through the epithelial layer into subjacent tissues. The particles passed between the epithelial cells, particularly in areas of desquamation. Persorbability was limited by size and hardness, hard particles in the range 7-70μm giving the best results although persorption still occurred in the size range 5-150μm. Mechanical factors are thought to be responsible for persorption as drugs which increased gastrointestinal motility also increased the rate of persorption, the reverse was also true for drugs that reduced gastric motility (58).

A mechanism for persorption was proposed by Luckey (63). As the villus contracts like a piston, the pressure pushes lymph from the lacteals into the lymphatic vessels. This adds to the pressure already being exerted on the epithelial cells by the reproducing cells around the base of the villus. Any loose cells in the extrusion zone of the villus are

forced off the basement membrane in a mini eruption. Relaxation of the villus produces a relative difference in pressure allowing a micro-implosion to draw particulate matter through the wound created by the discarded epithelial cells. This mechanism agrees with the theory proposed by Volkheimer, that persorption occurs particularly through areas of desquamation, such as at the tips of the villi.

It was suggested that persorbed particles were transported mainly via the lymphatic system with some portal transport. The distribution of particles between the two routes is likely to depend on particle size with the smaller particles being carried mainly by portal circulation and the larger ones by the lymph (61). The same study showed that persorption decreases with increasing age of the animal under test. Various peaks of persorption were demonstrated after particular age intervals.

Many scientists consider this line of research to be very limited, since it deals with a passive process and not an active one. The results quoted have not been accepted by all researchers.

More recently the accumulation of certain inert particles in mouse Peyer's patches (PP) after ingestion has been demonstrated. Chronic experiments, in which mice were fed carbon (20-50nm), or latex (2μm) in their

drinking water led to the uptake and retention of particles in PP (64-67) . Absorbed particles reached all parts of the PP follicle, including the germinal centre and were accumulated in macrophages. The macrophages were thought to be responsible for the transport of particles throughout the PP (67-70). However, there was little evidence of transport of particles from the PP to the bloodstream. In another study, 5.7μm and 15μm particles were not found in blood or reticuloendothelial organs up to four hours after ingestion, although some 5.7μm particles were recovered from the mesenteric lymph nodes and the lungs after long term ingestion (64). It has been suggested that particles which bypass, or are released from, mesenteric nodes are trapped in the lungs and that some are carried into the airways by pulmonary macrophages and excreted via the mucociliary "elevator system" (71).

The uptake of 2μm latex particles has been shown to occur even in the complete absence of bacteria, this demonstrates that bacterial activity is not necessary for uptake (72). It has been suggested that surface characteristics as well as particle size governs accumulation in PP, with hydrophobic particles being taken up more easily than hydrophilic ones (71,73).

In healthy human volunteers <u>Candida albicans</u> cells were shown to penetrate the intestinal wall following oral administration, probably by the mechanism of persorption, and to reach the blood and urine (74). Further studies in dogs and primates confirmed that <u>Candida albicans</u> cells are persorbed across an intact intestinal mucosa (75). Persorption of living candida may account for certain cases of systemic candidiasis which have followed oral administration of large doses of antibiotics.

It is evident that particulate material may be absorbed across the gastrointestinal tract and that the PP is a less effective barrier than the rest of the epithelium. The precise mechanisms involved in this process and the fate of the absorbed particulates have not yet been clearly elucidated.

1.5. Gut associated lymphoid tissue (GALT).

The gut contains as much lymphoid tissue as the spleen and probably synthesises more immunoglobulin than any single organ. The gut associated lymphoid tissue is distributed in four recognised anatomical locations, a) the lamina propria, which contains many cell types, a large proportion being IgA-secreting plasma cells, b) the intraepithelial lymphocytes, dispersed between the epithelial cells of the mucosal

membrane, c) the isolated lymphoid follicles, which are present throughout the small intestine and colon, and d) the PP, which are clusters of lymphoid follicles also found along the length of the small intestine. The lymphoid tissue in the lamina propria and the intra-epithelial lymphocytes are known as the diffuse lymphoid tissue (76). The lymphoid tissue responsible for the initiation of the immune response is found in both the isolated lymphoid follicles and the Peyer's Patches.

1.5.1. Diffuse lymphoid tissue

The lamina propria contains large numbers of macrophages, neutrophils, eosinophils, plasma cells and mast cells. These cells are all absent at birth but gradually accumulate during the first few weeks of life probably in response to extrinsic antigens. The cells in the lamina propria are thought to regulate the function of the overlying epithelium through immunological reactions (77).

The epithelium lining the gastrointestinal tract contains up to 20% of non-epithelial cells, most of which are intraepithelial lymphocytes (IEL). In the stomach and proximal small intestine over 95% of IEL are T lymphocytes, whereas in the colon and rectum 85-95% are T lymphocytes and the remainder are B

lymphocytes. The role of the IEL in the mucosa is

unclear but their numbers are greatly reduced in germ

free animals. It is probable that they are activated

lymphocytes produced by a cell mediated response to

intraluminal antigens, and they may play an important

role in processing intraluminal antigens (78).

T lymphocytes appear capable of crossing the basement

membrane in both directions (79). They may transport

antigens within the cell or bound to the cell surface.

Owen, Bhalla and Apple (80) observed morphologically

intact lymphoid cells migrating through discontinuities

in the epithelium produced by dead and damaged

epithelial cells. They also observed lymphocytes

extending through pores surrounded by intact M-cells

maintaining tight junctions with adjacent enterocytes,

but were unable to identify lymphocytes migrating from

the lumen back into the epithelium.

1.5.2. Peyer's patches (PP).

Lymphoid follicles grouped in the submucosa of the

small intestine and devoid of afferent lymphatics are

called Peyer's patches. Each lymphoid follicle has a

dome like appearance on the luminal surface (76). The

distribution of PP varies between species, in man they

are more common in the ileum (81). The epithelium

covering PP usually lacks goblet cells and is made up
of columnar cells interdigitating with M-cells (82).

The lymphoid tissue of the PP forms four anatomically
separate areas a) the follicle, which contains a
germinal centre, b) the dome overlying the follicle, c)
a thin rim of cells below the germinal centre, and d)
an interfollicular area (Figure 1.3.). The adult PP
consists of 40-70% B lymphocytes and 11-40% T cells
which are differentially distributed. The B lymphocytes
are found primarily in the germinal centre, whereas T
cells predominate in the dome and interfollicular
areas. This distribution results from the different
migratory patterns of the cells. T cells migrate
preferentially to the peripheral lymph nodes, whereas B
cells demonstrate a relative preference for PP and the
spleen. This specific localization is independent of
the lymphocyte source and is determined by recognition
of organ specific markers on the endothelial cells of
high endothelial venules (83).

There is evidence to suggest the presence of duct
associated lymphoid tissue in the minor salivary
glands, similiar to the GALT, which is accessible via
the salivary ducts (84). Perhaps this indicates that
local stimulation is important, particularly for
salivary immune responses.

VILLI

LYMPHOID FOLLICLE

epithelial cells

dome of cells containing M cells
dispersed amongst epithelial cells

mature and
immature M cells

Interfollicular area
(T cell area)

goblet cells

germinal
centre
(B cell area)

thin layer of cells

crypts (M cells differentiate here)
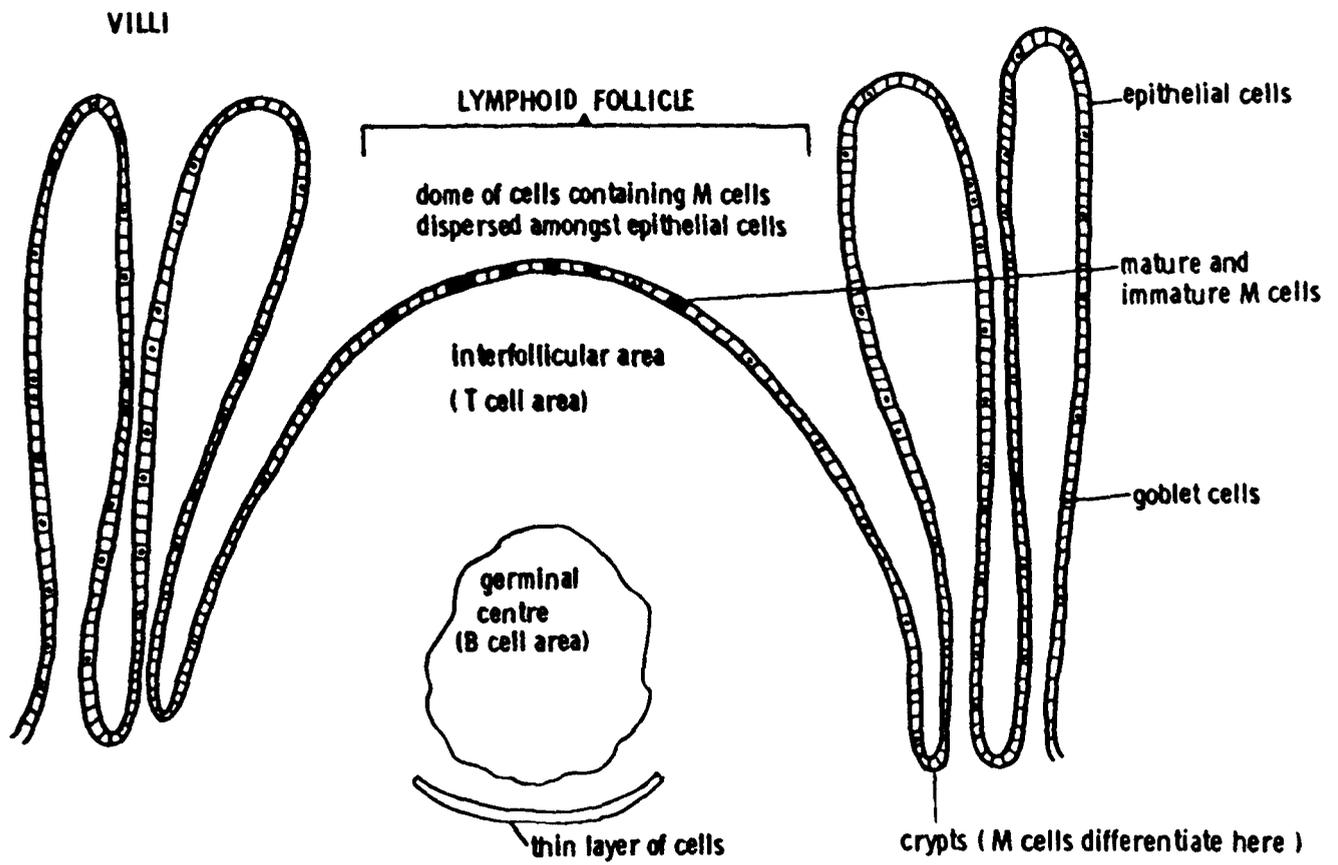
Figure 1.3. Schematic diagram of an intestinal lymphoid
follicle.

1.6. M-cells.

Specialised epithelial cells called M-cells, are
found interdispersed amongst the other epithelial cells
of the lymphoid follicle dome (85). M-cells are
modified to absorb antigens from the gastrointestinal
lumen and to deliver them to the lymphoid tissue where
an immune response is stimulated. Clusters of M-cells
have been identified in many species overlying the
lymphoid follicles in the gut (85-87); similar cells
have been found in other areas. Lymphoepithelial cells
with characteristics similiar to intestinal M-cells are
found in the epithelium overlying aggregated nodular
lymphoid tissue in the respiratory tract (88-90).
Bockman and Cooper (87) demonstrated uptake of ferritin
into the Peyer's Patches of mouse intestine, the Bursa
of Fabricius of chickens and the appendix of rabbits.
M-cells have also been called follicle associated
epithelial cells due to their general association with
lymphoid follicles (91).

M-cells are both functionally and morphologically
different from columnar epithelial cells (82). If cell
specific markers can be identified on the M-cells it
may enable delivery of macromolecules to a site
specifically developed for the absorption of such
molecules. It seems unlikely that M-cells operate as

antigen presenting cells. It has been suggested that
ordinary enterocytes can function as antigen presenting
cells (92,93), but this is unlikely to be important
physiologically since the ordinary enterocytes are
isolated from lymphoid cells. M-cell function is
probably more aptly described as antigen sampling.

1.6.1.Function

M-cells transport many macromolecules and
micro-organisms from the gastrointestinal lumen to the
underlying lymphoid tissue (Table 1.2.). It is not
known whether M-cells act as passive conduits for these
antigens or whether they process the antigens in some
way.

A clathrin-like coated pit has been demonstrated on
some endosomes of M-cells suggesting that a type of
receptor mediated transport is involved (94), but the
bulk uptake of markers such as HRP and ferritin
indicate that not all uptake is receptor mediated. HRP
has been shown to bind to the surface of M-cells
entering the cells via a pinocytic process forming
apical vesicles (Figure 1.4.). It is transported across
the cell within the vesicles and released, by
exocytosis, into the extracellular space between the
M-cells and the enfolded intrusive cells in the central
hollow (see below). The HRP then binds to the surface

MACROMOLECULES	MICRO-ORGANISMS	
Native ferritin	Viruses –	Reovirus type 1
Cationised ferritin		Reovirus type 2
Horseradish peroxidase	Bacteria –	Mycobacteria
Ricinus communis agglutinin II		Chlamydia
Wheatgerm agglutinin		Vibrio cholera

Table 1.2. Materials known to be transported by
M-cells.
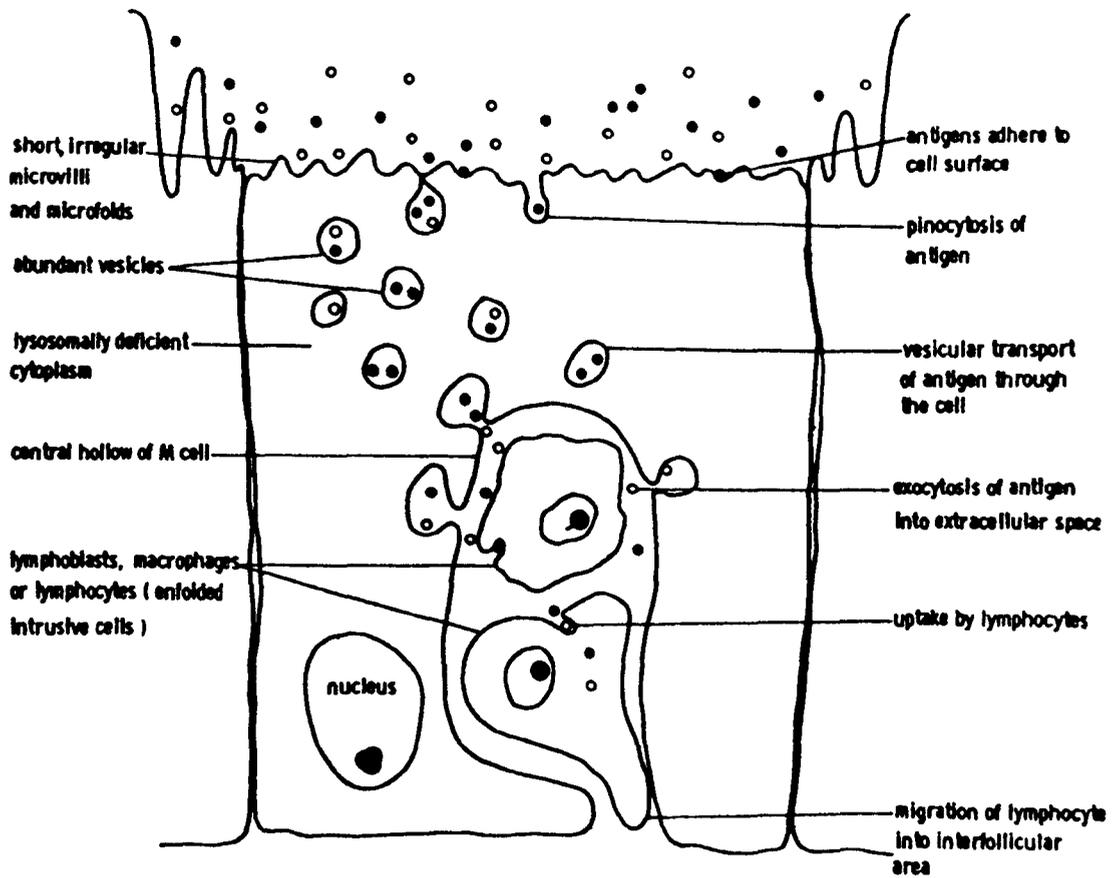
Figure 1.4. Schematic diagram illustrating the process by which antigens are absorbed via M-cells.

of, and is subsequently taken up by, lymphocytes and macrophages (86).

Histochemical studies have demonstrated a preferential uptake of HRP into intestinal M-cells when low concentrations of HRP are present. If larger amounts of HRP are present a more generalised uptake by all epithelial cells occurs by the mechanisms described previously (section 1.3.2.). This suggests that the mode of antigen uptake and penetration in the gut may depend on the antigen concentration present. At physiological, or lower levels of lumenal antigen, the specialized uptake pathway is preferred, whereas at higher antigen levels a more generalized uptake of antigen takes place. The mechanism of specialized antigen uptake by the gut seems to provide an important specific access route for intestinal antigens to reach lymphoid tissues and thereby stimulate the local immune system.

Materials much larger than HRP are also transported by the M-cells. It has been shown that this is the site where reoviruses penetrate the intestinal epithelium (95,96), <u>Reovirus type 1</u> adheres specifically to M-cells. M-cells have also been shown to be capable of phagocytosing and transporting intact <u>Vibrio cholerae</u>, which are curved rods of size 1.5 by 3µm (97,99). The increased capacity of M-cells to take up and transport

various substances has not been fully explained. The thinner glycocalyx over the cells may simply make them more accessible than ordinary enterocytes, or perhaps the M-cell surface is more sticky due to the presence of polyvalent adhesins. This latter idea is supported by the fact that some micro organisms bind preferentially to M-cells (95,99,105).

1.6.2. Morphology.

M-cells display a number of characteristic features which distinguish them from neighbouring cells. The surface lacks glycocalyx (98,100) and is characterised by microfolds and short irregular microvilli that are often wider and fewer in number than those on absorptive cells. The cells are cubical in shape rather than columnar (82). Vesicles are abundant in the apical cytoplasm and M-cells have been shown to endocytose and transport macromolecules (85,87,101,102) and micro-organisms (95,97,99,103) through the cell. The mitochondria are more electron dense than those in other cells and they are located in the apical region of the cytoplasm, which correlates with the transport function of the M-cells. There is also a deficiency of lysosomal material, but other organelles in M-cells are similiar to those in surrounding absorptive cells (104). The mature M-cell has a thin bridge of apical

cytoplasm and a basally located nucleus. Its cytoplasm
surrounds one or more intrusive cells, which are
usually lymphocytes (86), lymphoblasts (98), or
macrophages (97,103), but occasionally plasma cells
(98) or polymorphonuclear leucocytes (105). These cells
are not within the M-cell cytoplasm, but are in the
intercellular space that indents the cytoplasm, forming
a 'central hollow' (80).

Various theories have been proposed to explain the
origins of M-cells. There is some evidence to support
the suggestion that the cells differentiate from mature
absorptive cells on the upper part of the dome
(86,98,106,107). However it is now generally accepted
that many, if not all, M-cells differentiate directly
from undifferentiated cells of the crypts encircling
Peyer's Patches (91). There are two major subsets of
M-cells, which have been termed mature and immature on
the basis of their differing structural features (108).
Although the subsets differ structurally, they are both
termed M-cells because they display the same binding
and functional characteristics, namely: selective
adsorption of reovirus type 1, internalisation of
intraluminally administered ferritin, absence of lipid
uptake from micellar solutions and apical membranes
rich in cholesterol (94,108). It has been postulated
that lymphocytes influence differentiation of M-cells

from immature to mature cells in the epithelium overlying the lymphoid follicles, as the mature cells seem to be closely associated with lymphoid cells (98,106).

The role of M-cells must be put into perspective relative to ordinary enterocytes, which are much more abundant and are also capable of the uptake of macromolecules. Material taken up into enterocytes will be extensively degraded by lysosomal enzymes during passage through the cell, whereas M-cell uptake is not followed by degradation. Also, material taken up by M-cells is very likely to be presented to immunocompetent cells because of the close association of these cells. Material absorbed by the enterocyte wiil gain access to the blood or lymph, where it is rapidly filtered out of the system. Therefore, the uptake of proteins by ordinary enterocytes may lead to the induction of a systemic immune response, whereas the uptake of proteins by M-cells may result in the induction of a secretory immune response.

Comparisons of the apical membranes of M-cells and absorptive cells have been made in attempts to explain their varying functions. Histochemical studies have shown that the apical membrane of the M-cell has less alkaline phosphatase activity, but more esterase activity, than absorptive cells (107,109). Various

ferritin labelled lectins, including ricinus communis

agglutinins I and II, concanavalin A and dolichos

biflorus, bind sparsely to both M-cells and absorptive

cells in organ culture. Whereas wheatgerm agglutinin,

which has an affinity for terminal sialic acid residues

and oligomeric N-acetyl-D-glucosamine, binds more

avidly to M-cells than to the surrounding absorptive

cells in unfixed tissue (110). In fixed PP the binding

is the same for M-cells and absorptive cells for all

lectins tested (109). Staining with ruthenium red has

confirmed that M-cells have a less elaborate glycocalyx

than absorptive cells (105). Exposure of fixed

intestinal tissue to the polyene antibiotic filipin,

which binds cholesterol in a 1:1 ratio, reveals an

abundance of filipin cholesterol complexes in the

M-cell apical membrane, except in areas involved in

endocytosis (110). M-cells have also been shown to have

a low protein to lipid ratio (94). Further studies of

the M-cell apical surface may help to explain how the

cells bind antigens prior to their uptake. This

knowledge would be of great benefit in attempts to bind

material specifically to M-cells and to promote their

subsequent uptake.

1.7. The immune response

The Peyer's Patches via the M-cells, allow the migrating lymphoid cells to come into contact with intestinally derived antigens. After absorption, antigens are likely to be endocytosed by macrophages in the PP. Most of this antigen will be degraded rapidly in the phagolysosomes, however, part of the antigen is then expressed on the macrophage surface and will be recognised by T helper cells. Macrophages are very important in the presentation of antigen to T helper cells, because of their ability to regulate expression of Ia (histocompatibility) determinants and to process both particulate and soluble antigens (111). Through this mechanism, an immune response to the antigen can be initiated. Primed lymphocytes pass from the PP into the submucosal lymphatics and drain via the mesenteric vessels into the thoracic duct. Partly matured lymphocytes return to the mucosal secretory sites, including the lamina propria where they mature and secrete immunoglobulins (Figure 1.5.).

Peyer's Patches contain modified post capillary venules that allow lymphocytes to escape into the lymphoid tissue. Efferent lymphatics surround Peyer's Patches which drain predominantly into the mesenteric nodes but a few vessels drain directly into the lamina

Intestinal antigen

through M cells

T and B   via post-
——————————— lymphoid follicle ————————▶ Primed lymphocyte – – – – small proportion via direct
lymphocytes   capillary   route to lamina propria
                  venules

efferent lymphatics

thoracic duct

systemic circulation _ _ _ possible involvement of
                                       other lymphoid organs e.g. spleen

mucosal sites of ———————————————— Lymphoblasts (partly matured)

common mucosal immune system

e.g. lamina propria and lung

secretory component or re-exposure
to antigen stimulates further maturation

Fully mature IgA secreting plasma cells
or effector T cells

Figure 1.5. Flow diagram of the sequence of events
involved in the immune response to intestinally
absorbed antigen.

propria. However there are no afferent lymphatic vessels from the lamina propria to the Peyer's Patch to allow passage of cells in the reverse direction.

The majority of lymphocytes reaching the thoracic duct are primed to produce IgA (IgA bearing lymphocytes). The B lymphocytes of the Peyer's Patches give rise mainly to IgA bearing lymphocytes because there is antigen induced isotype switching of the B cells in situ (112). The switch T cells in the Peyer's Patches operate as true switch cells, controlling DNA recombination events, rather than as classical helper cells which act to expand already differentiated cells (113). The B cells mature into plasma cells (IgA secreting cells) only when they leave the restrictive environment of the Peyer's Patches. It is not known how the Peyer's Patches provide a special milieu in which isotype switching without terminal maturation of the B lymphocytes to plasma cells can occur, but the T suppressor cells in the Peyer's Patches certainly have a role to play here.

The primed lymphocytes may undergo further processing in other lymphoid organs such as the spleen. The partly matured plasma cells (lymphoblasts) return to the lamina propria or other mucosal sites and then differentiate into fully mature IgA secreting plasma cells or effector T cells. There appears to be some

component of the environment which causes lymphoblasts to selectively mature at the mucosal sites. It has been suggested that the secretory component (described below) or re-exposure to the lumenal antigen may be important in this process. Experimental evidence indicates that antigenic re-stimulation of the primed lymphoblasts is particularly important in the salivary gland (114). B and T cells, primed in the gut lymphoid follicles, migrate to mucosal surfaces in many locations in the body including the gastrointestinal tract, lung, breast, cervix, uterus and salivary and lacrimal glands. This has led to the concept of the common mucosal immune system (115).

The main immunoglobulin produced from the plasma cells of the lamina propria is IgA, but IgM, IgG, IgD and IgE cells are also found. IgA is found in the serum, but is mainly found in the secretions of all mucosal sites in the body. In animal species it is mainly dimeric, consisting of two IgA monomers bound together by a J chain, the IgA monomer and J chain are both synthesised by submucosal plasma cells. In humans, secretory IgA is dimeric, but 90% of serum IgA is monomeric. It is possible that in humans both monomeric and dimeric IgA is synthesized in the intestine and then diffuse or are transported either into the serum and to the mucosal surfaces. The secretory component

synthesised by the glandular epithelial cells is also found at the mucosal site. This transports IgA across the epithelial cells and protects it from proteolysis in the gut lumen.

Secretory immunoglobulins complexed with the glycocalyx on the surface of the intestinal epithelium forms a protective barrier against the penetration of micro-organisms (116). Binding to the gastrointestinal mucosa is a prerequisite for bacterial colonization. Secretory IgA combines with the bacterial antigens responsible for colonization and thereby helps to prevent it (79). Secretory IgA is effective at agglutinating bacteria because of its multiple valency, but it does not fix complement activated via the classical pathway. Several studies have shown a strong correlation between the presence of antibody in secretions and resistance to viral replication at mucosal surfaces.

Local immune mechanisms may provide a control mechanism for limiting the intestinal absorption of intact macromolecules. Immune exclusion may protect the host from excessive absorption of many macromolecules by forming non-absorbable complexes on the mucosal surface, which may then be degraded (79).

In rats dimeric IgA is rapidly transported from serum into bile via a saturable transport process. This

uptake mechanism is associated with the presence of secretory component in the hepatic parenchymal cells, which appears to act as receptor for the IgA (117,118). A similar mechanism also occurs in humans. At certain stages of lactation IgA is selectively transported from serum into ruminant milk via a transport mechanism dependent on the presence of secretory component in the mammary glands (119). These observations indicate that secretory component dependent removal of IgA from serum may be a feature of all mucosal sites. This could provide a compensatory mechanism to ensure that IgA reaches mucosal secretions even in the absence of local production.

The secretory immune system of the body is thus adapted to prevent the uptake of large amounts of protein at mucosal surfaces. The presence of this immune response mechanism within the gastrointestinal tract may have a considerable effect on the chronic delivery of proteins via the oral route and requires further investigation. However it does raise the possibility of oral immunisation against pathogens, particularly against those which enter the body by the enteric route or proliferate in the gut. This may provide an attractive alternative to parenteral immunisation and may indeed be more appropriate for such organisms. Oral immunisation may also elicit

protective antibodies against organisms which infect

other mucosal sites, such as the respiratory or the

genital tract.

Oral immunization alone would have several

advantages over parenteral or combined therapy. Oral

vaccines would be easy and inexpensive to administer

and because of the relative absence of side effects

would be far more acceptable to patients. The vaccines

would not need to be highly purified, which would

simplify preparation and in certain cases, eg. if the

immunogenicity was enhanced by formulation factors, a

killed organism might be appropriate, which would

eliminate the difficulties of developing an attenuated

strain.

1.7.1. Attempts to enhance the immune response.

A number of workers have considered methods of

promoting the immune response to orally administered

antigens. In 1969, it was suggested that the soluble or

particulate nature of the antigen may affect the

secretory antibody response produced after oral

administration (160). Ebersole and Molinari

demonstrated that oral immunisation with bacterial

particulate antigens induced greater salivary antibody

responses than the corresponding soluble antigens

(161). More recently, Cox et al. have confirmed that

greater secretory immune responses can be induced by oral immunisation of particulate as opposed to soluble antigens, but they only determined immune responses to a small molecular weight hapten (2,4-dinitrophenol or DNP) (159,163). They did not attempt to assess immune responses to the carrier protein. The soluble form of the antigen was more effective at priming parenterally for a subsequently enhanced secretory response than the particulate form (162). The greater secretory response after oral administration of particulate antigens may be due to a greater ability of particulate material to gain access to the tissue of the Peyer's Patch (95,137), to the greater uptake of particles by macrophages and other cell types after penetration of the PP, or to the persistence of the particles in the PP tissue after uptake (71). Soluble antigens are taken up to some extent by M-cells (74), but it has been suggested that a greater proportion of the soluble antigens will pass through the gut lamina propria into the venous system and initiate a systemic antibody response (164). It has also been suggested that the use of particulate bacterial antigens may result in an enhanced systemic response rather than tolerance as usually occurs with soluble antigens (148).

As was mentioned in section 1.2.2. and will be discussed further in chapter 5, several studies have

indicated that liposomes are capable of enhancing the secretory immune response to orally administered antigens (25-28).

1.8. Research aims.

The overall aim of this project was to enhance the immune responses to a chosen model protein following oral administration. To achieve this, the protein was incorporated into, or adsorbed onto colloidal carriers. We were particularly interested in the secretory immune response, but in all experiments the serum response was assessed also. If the model protein can be protected from degradation in the gut and its uptake into, and transport across, gut epithelial cells can be enhanced, then the concentration of intact protein reaching the serosal side of the intestine is likely to be increased. This is likely to result in an enhanced serum immune response. If the amount of protein that gains access to PP tissue can be increased and the presentation to macrophages, lymphocytes and dendritic cells can be facilitated, then an enhanced secretory immune response is likely. The literature evidence indicates that PP uptake, macrophage presentation and immunogenicity will all be enhanced by rendering the protein particulate. Evidence suggests that the incorporation of proteins into colloidal carriers may

confer some protection on the proteins against degradation in the gut.

The first objective of this work was to induce a significant positive salivary immune response and to demonstrate that the chosen immunoassay technique, an enzyme linked immunosorbent assay (ELISA), could detect a positive response in the saliva. Prior to this of course, the ELISA technique itself needed to be validated for the detection of antigen specific rat IgA and IgG. In early efforts to produce a significant positive salivary immune response, against DNP haptenated species, horse heart myoglobin (MYO) was chosen as the model protein carrier. It was chosen because of its proven ability to form a stable conjugate with DNP. However, due to the poor solubility of this conjugate, MYO was subsequently replaced with bovine gamma globulin (BGG).

In later work involving oral administration, the DNP haptenated species were replaced by native protein models, since we wished to be able to determine if major structural or conformational changes of the protein ocurred during formulation. MYO was used in the early oral immunisation studies, but poor solubility limited its incorporation into the chosen formulations. Therefore, it was subsequently replaced by ovalbumin (OVA); this protein is cheap, soluble and is widely

used in immunological research. Since this material is isolated from eggs, it will not be contaminated with any extraneous immunoglobulins which might interfere in immunoassay procedures.

The main objective of this project was to select several colloidal carriers as candidate pharmaceutical formulations which offered promise as orally administered immunological adjuvants either by virtue of their ability to confer protection on an entrapped protein and/or their ability to facilitate uptake by M-cells. Particles varying in size from 0.05µm, carbon (72), to 2µm, latex (73), have been shown to accumulate in PP during chronic feeding and organisms as large as 1.5µm by 3µm have been shown to be transported by M-cells (97,99). It is not known which size of particulate might be expected to gain better access to the PP tissue. Polyacrylamide beads of about 1-3µm, after oral administration, have been shown to induce better secretory immune responses to material adsorbed to their surface than an equivalent dose of soluble material (159,163). Therefore, colloidal carriers which could be prepared at or below this size were selected for oral immunisation studies. Immunisation protocols and immunoassay procedures needed to be developed to generate reliable and reproducible levels of salivary antibody in the rat and to allow their quantitative

determination. The model protein needed to be incorporated into the chosen formulations and assays had to be developed to determine the extent of this incorporation. The effectiveness of the formulations as orally administered adjuvants was then assessed.

2. ASSAY METHODS.

This chapter describes the immunoassay procedures adopted in attempts to determine salivary and sera immune responses to the model protein and the assay procedures adopted to determine the extent of its incorporation into, or adsorption onto the colloidal carriers. A method is also described which allowed us to demonstrate that the antigenicity of the model protein was unaltered by its association with the carriers.

2.1. Enzyme linked immunosorbent assay.

2.1.1. Introduction.

An assay technique was required to determine antibodies in rat saliva and sera samples in order to assess quantitatively the effectiveness of the formulations as orally administered immunological adjuvants. A 'sandwich' type direct binding immunoassay was performed in which different isotypes of antibody in the rat samples competed for binding sites on the immobilised antigen and a second, enzyme-labelled, anti-antibody was used to determine the primary antibody titre.

Enzyme labelled reagents are said to have several advantages over their equivalent radio-isotope labelled

counterparts, including long term stability, reduced health hazards, no licence required for use, no difficult safety precautions, easy disposal and simple inexpensive equipment required for read-out. However, enzyme labelled assays are not generally as sensitive as the radio labelled counterparts.

Two groups of experimental animals were immunised with the chosen model antigens by intraperitoneal (i.p.) injection with a known adjuvant, Freund's complete adjuvant. The presence of both IgA and IgG anti-OVA antibodies in the sera was then demonstrated by an ELISA. An assessment was made of the reproducibility of the assay system by performing repeated assays on the same serum sample on different days.

2.1.2.Materials and apparatus.

Freund's complete adjuvant (FCA) - Sigma, Poole, Dorset.

Horse heart myoglobin type III - Sigma.

Sheep anti-rat IgA horseradish peroxidase conjugate - Serotec. Oxon. England.

Sheep anti-rat IgG horseradish peroxidase conjugate - Serotec.

Ovalbumin (OVA) - Sigma Grade III.

Bovine serum albumin (BSA) - Sigma.

2,2'-azino-di-(3-ethylbenzthiazoline sulphonate) (ABTS)

- Amersham, Bucks, UK.

Microtitre plates PVC - Flow Laboratories,

Rickmansworth, Herts.

Titertek Multiskan - Flow Laboratories.

2.1.3. Methods.

2.1.3.1. Immunisation protocols.

(i). 500μg of myoglobin in 0.5ml FCA was injected into each of six rats by i.p. injection; sera samples were collected 10 days later.

(ii). 500μg of ovalbumin in 0.5ml FCA was injected into each of six rats by i.p. injection and sera samples were collected 14 days later. Another 14 days later, these rats received a booster dose of 100μg ovalbumin, also in FCA, by i.p. injection; sera were collected 7 days later.

2.1.3.2. Immunoassay.

(a). 50μl of ovalbumin at 10μg/ml in carbonate buffer pH 9.5 was incubated overnight at 4°C on microtitre plate.

(b). The plate was washed three times in phosphate buffered saline pH 7.2 (PBS) containing 0.1% Tween 20.

(c). 100μl 1% BSA was added to the plates and incubated at room temperature for 30 minutes, then discarded.

(d). The primary antibody, rat sera or saliva, was added at an appropriate dilution and incubated at 37°C for 1 hour.

(e). The plate was washed three times in PBS/0.1% Tween 20.

(f). 50μl peroxidase linked second antibody, at appropriate dilution, was added and incubated at 37°C for two hours. Dilutions of 1/400 for IgA conjugate and 1/800 for IgG, both in PBS/0.1% Tween 20/1% BSA, were found to be appropriate.

(g). The plate was washed three times in PBS/0.1% Tween 20.

(h). 100μl ABTS substrate was added to each well.

(i). The optical density was read at 405nm on Titertek multiskan 1 hour later.

2.1.4. Results.

All ELISA results are quoted as optical density values at 405nm for 1/20 dilutions of sera samples, unless otherwise stated. The blank group included in table 2.1. received identical treatment to the immunised animals, but the immunising antigen was omitted. In immunisation protocol(ii), the same group of animals was repeatedly sampled, including

pre-inoculation. This work was performed at Beechams Bioscience Research, Epsom, Surrey.

There was considerable variation in the responses from different members of the same group of animals (table 2.2). In order to determine if this was due to genuine differences in responses from the animals, or due to variations in the assay, the same pooled sample from immunisation protocol (ii) 35 day collection, was repeatedly assayed for both IgA and IgG. The results are shown in table 2.3. and showed that the variations were due to genuine differences in response from the animals.

In order to determine if values from assays performed on different days could be directly compared, the same sample from immunisation protocol (ii) 14 day collection, was repeatedly assayed for IgG on four different days (table 2.4.).

The results showed that the interday variation was such that data obtained from assays performed on different days could not be directly compared.

Table 2.1. ELISA sera response following
intraperitoneal injection of myoglobin (mean ±SD n=6).

	IgA	IgG
Blank group	0.172 ±0.020	0.255 ±0.016
Immunised group	0.203 ±0.005	0.669 ±0.342

Table 2.2. ELISA sera response following
intraperitoneal injection of ovalbumin (mean ±SD n=5).

	IgA	IgG
Pre-inoculation	0.533 ±0.085	0.927 ±0.094
14 day	1.065 ±0.429	2.830 ±0.564
35 day	1.720 ±0.385	3.154 ±0.502

Table 2.3. ELISA sera response following intraperitoneal injection of ovalbumin, repeated assay of pooled serum sample collected day 35 (mean ±SD n=6).

Dilution	IgA	IgG
1/20	3.166 ±0.033	3.345 ±0.012
1/1280	1.382 ±0.201	3.289 ±0.021

Table 2.4. ELISA serum response following intraperitoneal injection of ovalbumin, repeated assay of the same sample on four consecutive days (mean ±SD).

Number of repeat assays per day on same sample.	IgG 14 day response
4	2.249 ±0.093
4	1.258 ±0.120
2	1.652 ±0.102
2	1.594 ±0.135

2.1.5. Discussion.

In all assays, the sera samples were diluted
stepwise down the plates, with an initial dilution of
1/20. For ease of interpretation, normally only the
results from the initial dilution is quoted, since
differences between groups are likely to be more
pronounced at the lowest sample dilution.

The ELISA system was capable of detecting both IgA
and IgG rat anti-OVA antibodies in sera following i.p.
injection of this protein with FCA. The assay appeared
sensitive, since 35 day sera from immunisation protocol
(ii) were still "positive" for both IgA and IgG at the
highest dilution assayed, 1/1280. There did not appear
to be a great amount of inherent variation in the assay
when one sample was repeatedly assayed (table 2.3). But
assays performed on different days were not really
comparable, since the assay values from the same sample
varied considerably (table 2.4.). We were advised that
this was a common problem with ELISA and that
generally, only samples run in the same assay can be
compared directly. In most subsequent assays, samples
from immunisation protocol (ii) 14 day response, were
used as positive controls. The 35 day response was too
great, since the Titertek Multiskan shows linearity
only up to an absorbance value of 2.000.

The assay seemed suitable for the attempted
determination of anti-OVA antibodies in the saliva of
rats. However, later results brought into question the
sensitivity of this assay (chapters 4 and 5).
Therefore, an alternative immunoassay technique, using
radioisotope labels, was established for the
determination of rat anti-OVA antibodies.

2.2. Radiolabelling with Iodine-125 and radioimmunoassay.

2.2.1. Introduction.

Two alternative radioimmunoassays were assessed for their potential to measure ovalbumin specific IgA and IgG antibodies. The methods utilised Staphylococcus aureus Protein A and swine anti-sheep IgG radiolabelled with Iodine-125 as developing reagents. Protein A is isolated from certain strains of Staph.aureus; it is a cell wall protein that binds selectively to the Fc region of immunoglobulin molecules, most notably IgG. The reactivity with the Fc region was classified originally as a non-specific "pseudo-immune" reaction, as opposed to the specific binding of antigen at antibody Fab binding sites (120).

Protein A reactivity is characteristic of IgG from most mammalian species, but there is significant quantitative differences in the extent of reactivity for different species. The extent of reactivity for many species has been quantified on the basis of a competitive inhibition assay (121). Rat IgG has only weak reactivity with Protein A, but the binding of sheep IgG is at least 2-3 times better. Although relative to some other species, such as human, rabbit

or pig, sheep IgG still has quite low reactivity with Protein A (122).

Protein A can in fact display three different types of binding activity; (a) the primary reaction with IgG and other immunoglobulin classes at sites located in the Fc region; (b) a similiar but apparently weaker binding located in the Fab region outside the antigen binding site; and (c) a typical antigen-antibody reaction involving Fab combining sites. For most practical purposes, the significant reaction is between Protein A and the Fc portion of IgG. Thus Protein A has become an extremely important immunochemical reagent and has been utilised in a wide range of radio- and enzyme- immunoassay procedures, see (121) for review.

The alternative radioimmunoassay technique involved a second anti-antibody, swine anti sheep IgG, which was also labelled with Iodine-125.

2.2.2. Materials and apparatus.

Staphylococus aureus, protein A - Pharmacia Chemicals AB, Uppsala, Sweden.

Swine anti-sheep IgG - Serotec, Oxford.

Sheep anti-rat IgG - Serotec.

Sheep anti-rat IgA - Serotec.

Iodine-125 (IMS 30) - Amersham International plc, Amersham, Bucks.

Chloramine T - Hopkins and Williams,

Sodium metabisulphite - Fisons, Loughborough.

Ovalbumin grade III - Sigma Chemicals, Poole, Dorset.

Bovine serum albumin grade V - Sigma.

Sephadex G-25, PD-10 column - Pharmacia.

Gamma counter - Packard 5610.

0.5M phosphate buffer - prepared by dissolving 7.1g

disodium hydrogen orthophosphate (A) and 7.8g sodium

dihydrogen orthophosphate (B) both in 100ml of

distilled water. The pH of solution A was then

monitored as solution B was added dropwise until the pH

reached 7.5.

2.2.3. Methods.

2.2.3.1. Staphylococcus aureus Protein A
radiolabelling.

Sodium Iodide-125 was oxidised by the powerful

oxidising agent chloramine-T to release Iodine-125,

which was then directly substituted into the aromatic

ring structures of the protein.

The purification step, column chromatography,

cleaned the product of unreacted iodine and some large

molecular weight aggregates brought about by

polymerisation.

(a). The column was first washed with PBS/Azide 0.02%, followed by 5ml 5% BSA and finally with 20ml PBS/Az.

(b). To the vial in which the IMS 30 was supplied, the following were added;

25μl Staph.Aureus at 1mg/ml in 0.5M phosphate buffer

25μl 0.5M phosphate buffer

25μl Chloramine-T 0.4mg/ml in 0.5M phosphate buffer

(c). The reaction mixture was mixed with a pipette tip and allowed to stand for 45 seconds.

(d). The reaction was quenched with 100μl 0.2mg/ml sodium metabisulphite in 0.5M phosphate buffer.

(e). The reaction mixture was then applied to the Sephadex PD 10 column along with 2x100μl potassium iodide 10mg/ml in 5%BSA/PBS washings from the reaction vial.

(f). The mixture was eluted with successive volumes of 0.5ml PBS and the fractions were collected in tubes containing 0.5ml 1% BSA/PBS.

(g). A 10μl aliquot from each fraction was then counted on a gamma counter and the fractions containing the peak activity were bulked.

(h). A 10μl aliquot of the bulked fraction was then counted for one minute to determine the activity present. The bulked Staph.aureus Iodine-125 solution

was then divided into suitable aliquots and stored

frozen.

2.2.3.2. Swine anti-sheep IgG radiolabelling.

The same radiolabelling method was used as described

for protein A, except the concentration of the protein

was altered. Swine anti-sheep IgG is supplied at a

concentration of 9.5mg/ml, but there is likely to be a

lot of other material present in the sample. Including

many immunoglobulins directed against totally unrelated

antigens. Therefore, this product was labelled at a

concentration of 4mg/ml since the extraneous material

present in the sample will also be labelled and

collected.

2.2.3.3. Radioimmunoassay protocol.

(a). 50µl of ovalbumin at 100µg/ml in PBS/Az, was left

in wells of microtitre plate overnight at 4°C.

(b). The plate was washed three times in PBS/0.05%

Tween 20.

(c). The plate was blocked by 1% BSA 100µl left on

wells for 30 minutes.

(d). Serum dilutions in PBS were added to wells and

incubated overnight at 4°C.

(e). The plate was washed three times in PBS/0.05%
Tween 20.

(f). The anti-rat immunoglobulins were added, diluted
in 1%BSA/PBS/0.05% Tween 20 at dilutions of 1/400 for
sheep anti-rat IgA and 1/800 for sheep anti-rat IgG.
They were incubated for two hours at 37°C.
Then either step (g)i. or (g)ii. was followed,
(g)i. 50μl Staph.Aureus Iodine-125 diluted in 1%
BSA/PBS/0.05% Tween 20 was added to each well and left
at room temperature for 3 hours.
(g)ii. 50μl swine anti-sheep IgG Iodine-125 diluted in
1% BSA/PBS/0.05% Tween 20 was added to each well and
left at room temperature for 3 hours.
(h). The plate was washed three times in PBS/0.05%
Tween 20, the individual wells were cut up and put into
individual tubes and the counts per minute value was
recorded by the gamma counter for each tube.

2.2.3.4. Assessment of radiolabelled reagents.

The rat sera were diluted across the plate, starting
at a dilution of 1/2. The assay was run in triplicate
for both radiolabelled reagents, which allowed a full
assessment of the suitability of the two reagents for
the detection of OVA-specific IgA and IgG antibodies.
Steps (A) to (E), described below, allowed a full
assessment of the contribution made to the overall

result by the non-specific binding of the second antibodies to normal rat sera and by the non-specific binding of normal sheep sera to "positive" rat sera.

The "positive" (+ve) rat serum was obtained from an animal which had been immunised by sub-cutaneous injection with 10mg ovalbumin in FCA. Serum was collected 18 days later.

(A). OVA + +ve rat serum + sheep anti-rat IgG + radiolabel

(B). OVA + +ve rat serum + normal sheep serum + radiolabel

(C). OVA + normal rat serum + sheep anti-rat IgG + radiolabel

(D). OVA + +ve rat serum + sheep anti-rat IgA + radiolabel

(E). OVA + normal rat serum + sheep anti-rat IgA + radiolabel

2.2.4. Results

The results from radiolabelling of Staph.Aureus Protein A and swine anti-sheep IgG with Iodine-125 are shown in tables 2.5. and 2.6. respectively. The total

activity present in the radiolabelled products was calculated as shown below.

2.2.4.1. Calculation of total activity present in radiolabelled products.

(i). <u>Staph.Aureus</u> Protein A.

The peak activity of radiolabelled protein was seen in the exclusion volume occuring in fractions 6-8, which were bulked. The unreacted iodine-125 started to come off the column at fractions 16-18. All the counts were taken over 15 seconds.

Three 20µl aliquots of the pooled fractions 6-8, were then counted for 15 seconds. The mean count obtained was 1327344.

The total activity present in the iodinated protein sample in µCi was calculated as follows;

$$\frac{\text{count from bulked fraction} \times C \times FV \times (1) \times (2)}{E \times (2.22 \times 10^6)}$$

where C = figure required to bring counting time to 1 minute.

FV = bulked fraction volume in ml.

(1) = figure to bring the aliquot volume to 1ml.

(2) = efficiency of the gamma counter

2.22×10^6 is a conversion factor to convert from

counts per minute to μCi.

Thus total activity present = 472μCi.

(ii). Swine anti-sheep IgG.

The peak activity of radiolabelled protein occurred

in fractions 6-8. Consequently, these samples were

bulked. All counts were performed over 15 seconds.

Three 20μl aliquots from pooled fractions 6-8 were

counted for 15 seconds. The mean count was 1156399.

The calculation was performed as above.

Thus total activity present = 411μCi.

2.2.4.2.   Radioimmunoassay.

The results from the assay involving swine anti-sheep

IgG-^{125}I, shown in table 2.8., were relatively easy to

interpret, with reference to the method described in

2.2.3.4. The c.p.m. values for the non-specific binding

of the normal sheep sera to the positive rat sera (B)

were subtracted from the values for the binding of the

corresponding dilutions of the second antibodies (A)

and (D). The values obtained after subtraction were

then compared to the values obtained for the

non-specific binding of these same second antibodies at

the same dilution to a normal (non-immunised) rat sera
(C) and (E). If these values were significantly greater
than the corresponding values for (C) and (E), then the
presence of OVA-specific antibodies would be
established.

The results from the assay for OVA-specific IgA
antibodies involving protein A Iodine-125, shown in
table 2.7., were also relatively easy to interpret.
Exactly the same subtractions and comparisons that were
carried out in the previous situation with swine
anti-sheep IgG also apply here. However, the results
for the assay of IgG antibodies using protein A (table
2.7.) were much more difficult to interpret, since
protein A binds not only to sheep anti-rat IgG, but
also to rat IgG. If the second antibody was in excess,
as it should be at a concentration of 1/800, then all
the rat IgG should be bound by a second antibody
molecule. However, the protein A may still bind to the
rat IgG, since it may interact at a completely
different combining site which may still be exposed.
Therefore, it is possible that the two reagents will
interact to amplify the result obtained for IgG.
Despite the complexities of interpretation, the assay
for OVA-specific IgG using protein A was still valid,
although the subtractions described earlier were no
longer necessary. The values obtained from positive and

normal sera were compared directly while ensuring that

the corresponding values from normal sheep sera were

not unreasonably high.

Table 2.5. Counts per minute for different fractions of
Iodine-125 labelled Staphylococcus aureus Protein A.

Fraction number.	Count.
1	17
2	26
3	19
4	17
5	221
6	201443
7	1955577
8	639990
9	64332
10	19412
11	11327
12	11704
13	12315
14	13227
15	16750
16	26051
17	82301
18	113701

Table 2.6. Counts per minute for different fractions of Iodine-125 labelled swine anti-sheep IgG.

Fraction number	Count
1	11
2	10
3	19
4	13
5	17
6	72471
7	1039865
8	413591
9	46189
10	18049
11	27190
12	19180

(A). OVA + +ve rat serum + sheep anti-rat IgG + radiolabel

(B). OVA + +ve rat serum + normal sheep serum + radiolabel

(C). OVA + normal rat serum + sheep anti-rat IgG + radiolabel

(D). OVA + +ve rat serum + sheep anti-rat IgA + radiolabel

(E). OVA + normal rat serum + sheep anti-rat IgA + radiolabel

Table 2.7. Radioimmunoassay results for Iodine-125 labelled Staphylococcus aureus Protein A, counts per minute (mean n=3).
See method 2.2.3.4. for full description of (A) to (E).

	(A).	(B).	(C).	(D).	(E).
DILUTION					
1/2	140125	29902	6692	134071	1743
1/4	103119	29511	7077	110179	2020
1/8	86432	30603	7158	78772	2319
1/16	65453	28936	5933	55837	2228
1/32	47633	25332	5236	42241	1686
1/64	33961	21077	4847	32510	1436
1/128	29295	17559	3827	26275	1344
1/256	27222	13452	3622	19360	2126
1/512	23853	9748	5114	15741	2495
1/1024	19587	7667	3558	10366	2359
1/2048	15608	5807	2788	7950	2180
1/4096	12872	4226	3219	5258	1496

Table 2.8. Radioimmunoassay results for Iodine-125 labelled swine anti-sheep IgG, counts per minute (mean n=3).

See method 2.2.3.4. for full description of (A) to (E).

DILUTION	(A).	(B).	(C).	(D).	(E).
1/2	12115	3287	11649	7054	6134
1/4	11412	2941	11500	9338	5579
1/8	11925	4240	10913	10805	4809
1/16	16118	5371	8381	8113	4568
1/32	18414	3881	6306	10034	4708
1/64	14392	6653	8030	7749	5133
1/128	15680	2256	2694	6037	4994
1/256	14590	4487	3267	7471	4396
1/512	14814	2719	3154	4996	2166
1/1024	13058	2714	3849	5974	4904
1/2048	14316	3124	3217	3941	3697
1/4096	8785	3295	2783	1565	3017

2.2.5. Discussion.

The assay method 2.2.3.4. allowed a full assessment of the effects of non-specific binding of the second antibodies to normal rat sera and of normal sheep serum to "positive" rat sera. Although normal sheep serum will not be identical to the material present in the highly processed second antibodies, it was thought that an assessment of the non-specific binding capacity of this reagent was necessary. The incorporation of normal sheep serum into the assay was the nearest approximation that could be achieved, since the precise processing of the second antibodies was unknown.

With radiolabelled swine anti-sheep IgG, the presence of OVA specific IgG antibodies in the "positive" rat sera was indicated. However, this particular assay system did not demonstrate the presence of OVA specific IgA antibodies.

With radiolabelled Protein A, satisfactory results were obtained, since both OVA specific rat IgA and IgG antibodies were detected. Non-specific binding was low throughout, both for the binding of the second antibody to the normal rat sera, and for normal sheep to "positive" rat sera. Therefore, the assay system involving radiolabelled Protein A was selected for further work.

2.3. Polyacrylamide gel electrophoresis.

2.3.1. Introduction

In order to demonstrate that the antigenicity of ovalbumin was unchanged by the adsorption to poly 2-butyl cyanoacrylate (p(BCA)) particles, or by the entrapment within liposomes, antibody responses to the formulated antigen should be assessed by assay against the native antigen. However, by performing polyacrylamide gel electrophoresis (PAGE) on the solubilised formulated antigen and immunoblotting against native OVA specific antibody, it is possible to determine whether the formulated antigen does have retained antigenic determinants.

Sodium dodecyl sulphate (SDS) PAGE is one of the most widely used methods for separating protein mixtures and for determining their molecular weight. SDS is an anionic detergent which causes denaturation and solubilisation of proteins. The efficiency of the solubilisation is due to the mutual electrostatic repulsion of the protein following the binding of a large number of detergent molecules on the surface. Proteins assume a rod shaped structure and the migration velocity shows an inverse linear correlation with the logarithm of the protein molecular weight.

Therefore, formulated ovalbumin was solubilized with SDS, electrophoresed on polyacrylamide gels and then blotted onto a nitrocellulose membrane (NC). The NC was incubated with rat sera containing OVA specific antibodies, prior to being probed with an anti-rat IgG horse radish peroxidase conjugate. Successful development of the immunoblot would confirm that anti-OVA antibodies in the rat sera still bound to formulated OVA.

Gels are produced when the acrylamide monomer is copolymerised with a cross-linking agent, usually N,N'-methylenebisacrylamide, in the presence of a catalyst accelerator chain-initiator mixture. This mixture will usually consist of freshly prepared ammonium persulphate as catalyst, together with N,N,N',N'-tetramethylenediamine (TEMED) as initiator. The porosity of a gel is determined by the relative amounts of acrylamide monomer and cross-linking agent used. The lower the percentage of acrylamide used, the larger the pore size present and so the lesser the resistance to the passage of large molecules.

2.3.2. Materials and apparatus.

Acrylamide, bisacrylamide and ammonium persulphate –
Bio-rad electrophoresis purity reagent, Bio-rad Labs,
Richmond, California, U.S.A.

Sodium dodecyl sulphate – 'specially pure', BDH
chemicals Ltd, Poole, England.

Tween 20 – Sigma Chemicals, Poole, England.

Trizma base– Sigma.

Bromophenol blue – BDH.

Mercaptoethanol – BDH

Glycine – BDH.

Coomassie brilliant blue R – Sigma.

Ponceau S – Sigma.

Ovalbumin – Grade III, Sigma.

Mini protean II cell – Bio-rad.

Power supply EPS 500/400 – Pharmacia AB, Uppsala,
Sweden.

SDS reducing buffer consisting of;

distilled water	4.0ml
0.5M Tris-HCl pH 6.8	1.0ml
glycerol	0.8ml
10% (w/v) SDS	1.6ml
2-mercaptoethanol	0.4ml
0.05% (w/v) bromophenol blue	0.2ml

Running buffer pH 8.3 consisting of;

Tris base   0.3%

glycine   1.2%

SDS   0.1%

2.3.3.   Method.

A discontinuous gel was cast consisting of a stacking
upper gel (4% acrylamide) and a resolving lower gel
(10% acrylamide). The stacking gel acts to concentrate
the samples and so results in better resolution in the
resolving gel. During the casting of the stacking gel,
a ten wedge comb produced the wells into which the
samples were introduced.

The following samples were electrophoresed after
solubilisation in SDS according to the method of
Williams et al (123):

(1). Native OVA solution.

(2). OVA encapsulated in liposomes.

(3). OVA adsorbed to p(BCA) particles stabilised with
dextran 70.

(4). OVA adsorbed to p(BCA) particles stabilised with
B-cyclodextrin.

Samples (3) and (4) were prepared as described in
6.3.1. and isolated by centrifugation. Unbound OVA was

washed off and the particles were resuspended in PBS
and stirred at room temperature overnight. The
liposomes were prepared as described in 4.3.1.

Aliquots of samples (1) - (4) were mixed with an
equal volume of reducing buffer and heated at 100°C for
ten minutes. Twenty microlitres of each cooled sample
was introduced into the sample wells using a Hamilton
pipette. This was performed in duplicate in two gels.
The cell was then run at 200V for about 45 minutes.

The gel was removed from the cell and placed in
Coomasie brilliant blue stain for 30 minutes, then left
in de-stain (40% methanol, 10% acetic acid) overnight.

2.3.4. Results.

A photograph of a typical polyacrylamide gel is shown
in figure 2.1.

2.3.5. Discussion.

The major protein components of the four OVA samples
described in the methods, 2.3.3., were electrophoresed
to the same level in the gel, indicating that ovalbumin
had not been degraded during its incorporation into
liposomes or its adsorption to p(BCA) particles.

The proteins were blotted from the gel to a
nitrocellulose membrane so that they could be probed
immunologically.

(1)   (2)   (3)   (4)
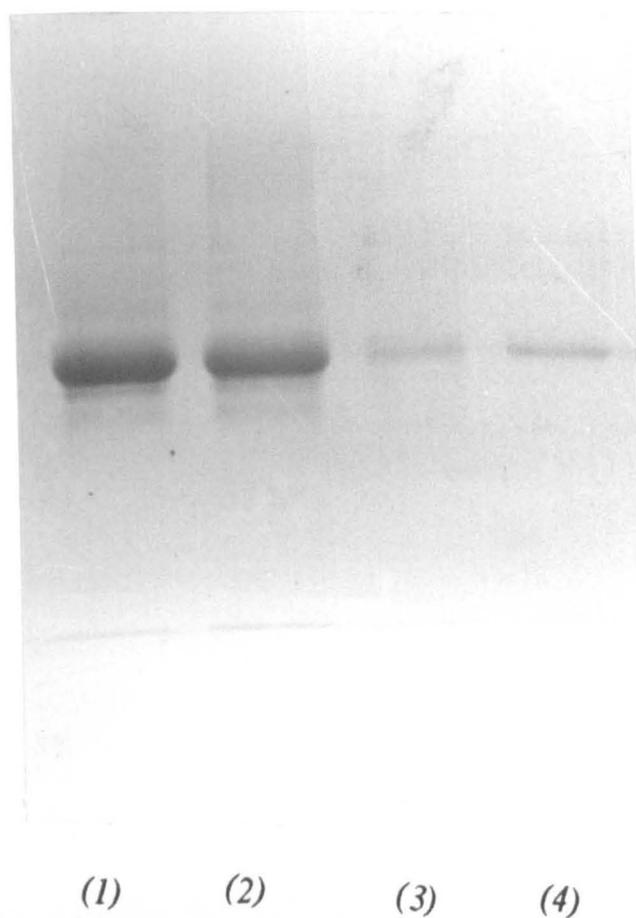
Figure 2.1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of (1) native ovalbumin, (2) ovalbumin incorporated in liposomes and ovalbumin adsorbed to (3) beta-cyclodextrin and (4) dextran 70 stabilised poly 2-butylcyanoacrylate particles.

2.4. Immunoblotting.

2.4.1. Introduction.

This section describes the blotting of ovalbumin from acrylamide gel to a nitrocellulose membrane and its immunological probing. Successful development of the immunoblot would confirm that the immune response to formulated ovalbumin can be assessed by immunoassay techniques which involve initial binding to the native antigen.

After separation of proteins in a gel matrix, staining is often limited by properties of the matrix itself. Thickness and non-uniformity affect the permeability to stains, while difficulty in handling and limits in durability restrict the use of multiple consecutive stains. These limitations are all lessened by the electrotransfer of proteins from the gel to a thin matrix, most commonly nitrocellulose paper. This is known as electroblotting or 'western blotting' and under optimal conditions over 90% of the protein is transferred.

For detection of proteins on nitrocellulose, the double antibody enzyme immunoassay system using horseradish peroxidase and 4-chloro-1-naphthol is generally recommended over all other systems because of

good sensitivity and negligible background development (Bio-rad technical literature).

The PAGE procedure as described in 2.3.3. was repeated, but the ovalbumin was not stained with coomassie blue, it was transferred to a nitrocellulose membrane.

2.4.2. Materials and apparatus.

PAGE - see section 2.3.2.

Sheep anti-rat IgG peroxidase conjugate - Serotec, Oxon.

4-chloro-1-naphthol - Sigma Chemical co., Poole, Dorset.

Trans blot cell - Bio-rad Labs, Richmond, California, U.S.A.

Nitrocellulose membrane - Bio-rad.

Power supply model 3000/300 - Bio-rad.

2.4.3. Methods.

2.4.3.1. Electrotransfer.

OVA was transferred from the gels by electroblotting according to the method of Towbin et al (124) and was detected by immunoblotting. The cell was run at 60V for about 5 hours, immersed in ice throughout to keep it cool.

The presence of the transferred protein was demonstrated with the reversible stain ponceau red, which was then washed off.

2.4.3.1. Immunological detection.

(a). The blot was soaked in 0.3% Tween 20 in saline/tris buffer (0.9% NaCl, 10mM tris HCl pH 7.4) for 1 hour at room temperature.

(b). It was then incubated with the first antibody, rat sera containing anti-OVA antibodies (positive control 5.5.1.), diluted 1/50 in 0.3% Tween 20/saline/tris for 4 hours at room temperature.

(c). It was then washed six times in tris/saline and incubated with the second antibody, sheep anti-rat IgG peroxidase conjugate, diluted at 1/1000 in 0.3% Tween 20/saline/tris for 2 hours at room temperature.

(d). It was then rinsed six times in saline/tris.

(e). The blots were then soaked in a solution of 25μg of 4-chloro-1-naphthol per ml in 0.01% hydrogen peroxide /10mM tris HCl pH 7.4. This solution was prepared fresh, with 4-chloro-1-naphthol being first dissolved in a small amount of methanol and then added to the solution containing the peroxide and tris.

(f). The reaction was terminated after about thirty minutes by washing the blot with distilled water.The blots were then photographed.

2.4.4. Results.

See section 2.3.4. for a photograph of a typical polyacrylamide gel. A photograph of the developed immunoblot is shown in figure 2.2.

2.4.5. Discussion.

Antibodies raised against native ovalbumin still combined with ovalbumin following incorporation into liposomes or adsorption to poly (2-butylcyanoacrylate) particles. Therefore the immune response to formulated ovalbumin can be reliably assessed by standard immunoassay techniques which involved initial binding to the native antigen eg. ELISA and RIA.
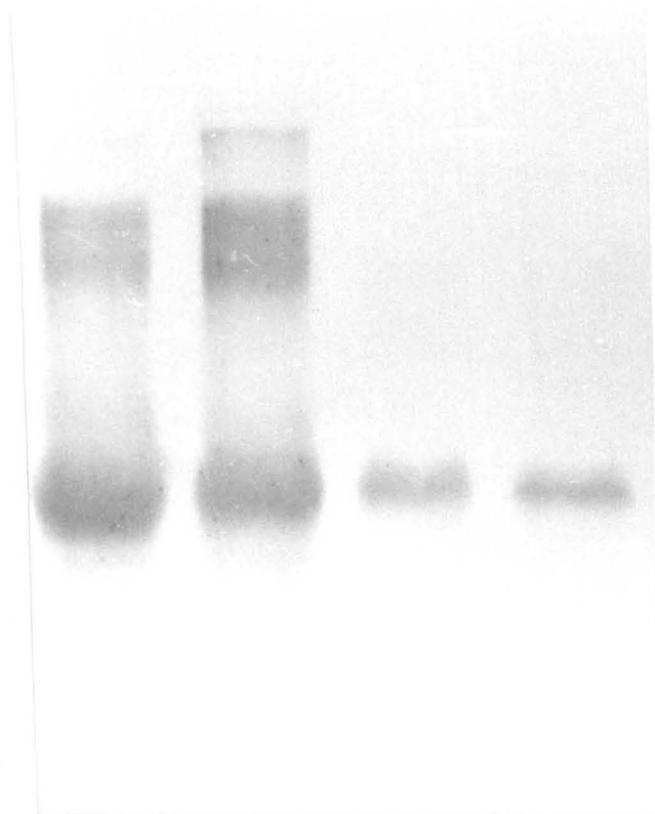
(1)   *(2)*   *(3)*   *(4)*

Figure 2.2. Developed immunoblot of ovalbumin samples
(1) - (4) described in legend for Figure 2.1. after
their electrotransfer to a nitrocellulose membrane.

2.5. Lowry assay.

2.5.1. Introduction.

Due to its simplicity, sensitivity and precision, the method of Lowry et al (125) is the most widely used assay for the quantitative determination of proteins. The method is based on the Folin-Ciocalteau phenol reagent, in which the active constituent is phosphomolybdic-tungstic mixed acid. Proteins cause a reduction of the mixed acid and produce one or more of several possible reduced species which have a characteristic blue colour. Copper apparently chelates in the peptide structure and facilitates electron transfer, thereby increasing sensitivity to protein. The principle chromogenic amino acids are tryptophan and tyrosine. Various modifications of this assay have been suggested (126). A modification which showed a more linear photometric response was employed in this work (127).

2.5.2. Materials and apparatus.

Folin-Ciocalteaus phenol reagent – Sigma.

Potassium sodium tartrate – BDH.

Sodium carbonate – BDH.

Copper sulphate – Sigma.

Kontron Uvikon 860 Spectrophotometer.

2.5.3. Method.

Solution A - 2g potassium sodium tartrate and 100g
sodium carbonate were dissolved in 500ml 1M NaOH and
diluted with water to 1L.

Solution B - 2g potassium sodium tartrate and 1g copper
sulphate wre dissolved in 90ml of water and 10ml of 1M
NaOH was added.

Solution C - (prepared daily) 1 volume
Folin-Ciocalteaus reagent was diluted with 15 volumes
of water.

1ml of the protein sample was treated with 0.9ml
solution A in a test tube and placed in a water bath at
50°C for 10 minutes, then cooled to room temperature.
It was then treated with 0.1ml solution B and left at
room temperature for 10 minutes before 3ml solution C
was added rapidly and mixed on a vortex within 1
second. The tubes were again heated at 50°C for 10
minutes and cooled to room temperature. The absorbances
were read in a 1cm cuvette at 650nm.

Unknown protein samples were compared with a set of
standards run concurrently in the assay.

2.5.4. Results.

To validate and characterise the assay, the same six standard solutions of ovalbumin were assayed on six separate days (table 2.9.).

2.5.5. Discussion.

The literature indicates that the Lowry assay is sensitive to the determination of proteins as low as $5\mu g$ (126). In our hands, the assay was sensitive to the determination of $25\mu g$ ovalbumin, lower amounts were not attempted since this was not required. However, variation in the absorbance values occurred when the same standards were assayed on different days. For the lower amounts of ovalbumin assayed, the standard deviations of the mean were about 10%, or more, of the total amount of ovalbumin present. This was considered to be an unacceptable degree of day to day variation in the assay. Therefore, when the assay was used subsequently, a set of standards were always run in the assay alongside the unknown samples for a direct comparison. The assay proved sensitive and precise for the accurate determination of ovalbumin, whenever required.

Table 2.9. Reproducibility of the Lowry assay (mean ±SD n=6).

Values quoted are absorbances at 650nm.

OVA (μg)	25	50	100	150	200	250
assay number.						
1.	0.092	0.151	0.300	0.420	0.585	0.688
2.	0.120	0.182	0.331	0.504	0.608	0.672
3.	0.120	0.188	0.342	0.479	0.650	0.729
4.	0.112	0.195	0.388	0.555	0.688	0.785
5.	0.100	0.192	0.327	0.511	0.637	0.721
6.	0.120	0.242	0.379	0.496	0.686	0.768
mean	0.111	0.192	0.345	0.494	0.642	0.727
±SD	±0.012	±0.029	±0.033	±0.044	±0.041	±0.044

2.6. Amino acid analysis.

An automatic amino acid analysis technique was required to determine the extent of entrapment of ovalbumin in polyacrylamide microparticles, because extensive hydrolysis of the particles was required to release the entrapped protein. This work was kindly performed on our behalf at Beechams Biosciences Research Division, Epsom, using the Waters PICO-TAG system. Three main steps were involved;

(1). Hydrolysis of the protein to yield free amino acids,

(2). Pre-column derivatization of the sample (Figure 2.3.)

(3). Analysis by reverse phase HPLC.

In the pre-column steps, protein samples were first hydrolysed with HCl, then derivatized with phenylisothiocyanate to produce phenylthiocarbamyl amino acids. These amino acid derivatives may then be analysed by HPLC in amounts as low as 1 picomole.
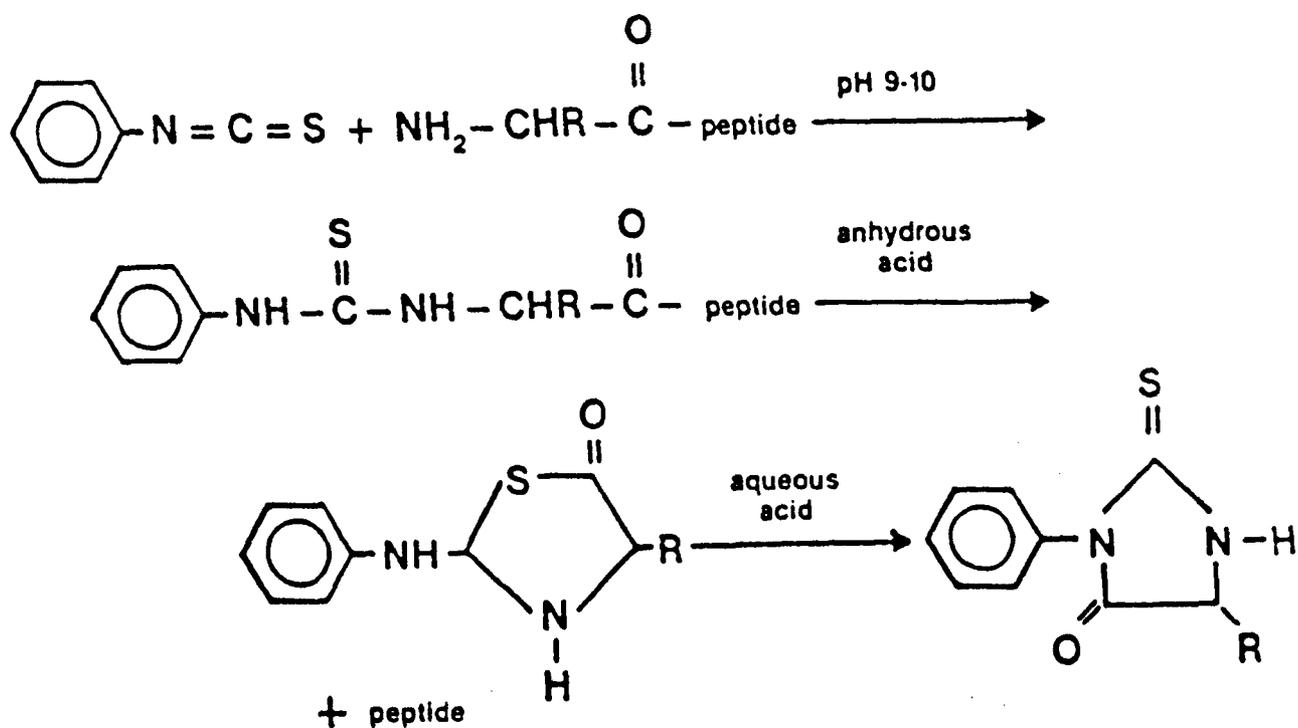
Figure 2.3. Phenylisothiocyanate derivatization of amino acids to produce phenylthiocarbamyl amino acids for analysis by high performance liquid chromatography.

3. IMMUNISATION PROTOCOLS.

3.1. Introduction.

A wide range of immune reactions may be elicited following oral immunization involving both the secretory and the systemic immune systems. The response to oral immunization seems to depend on a number of variables such as antigen type, dose, frequency of exposure and prior immune status of the individual. Other factors such as species, strain and age on exposure will also play a part.

Oral immunisation may give rise to antibodies, mainly IgA, in the gastrointestinal tract (128) and at distant sites such as mammary glands (129), lacrimal glands (130), or salivary glands (131). This occurs via the "common enteromucosal secretory antibody system". Enteric administration of antigens to unprimed animals can blunt the immune response to subsequent parenteral challenge with that same antigen, i.e. systemic tolerance (128,132-136) (Figure 3.1.). Such systemic hyporesponsiveness is antigen specific and will not develop if the animal has received prior systemic exposure to the antigen. Systemic tolerance appears to be induced via a suppressor circuit initiated in the gut associated lymphoid tissue upon exposure to antigen. A T suppressor-inducer cell (Tsi) resides in

Figure 3.1. - A simplified flow diagram illustrating
the simultaneous processes of systemic tolerance and
local antibody production. Antigens enter through the M
cells and interact with the B lymphocytes to produce
primed B lymphocytes (BAprimed) which then pass through
the mesenteric lymph nodes (M.L.N.) and seed out to all
secretory sites where they produce antibody. T helper
cells (T_h) interact with the B lymphocytes and assist
the priming process. T switch cells (T_{swi}) also present
in the Peyer's Patches isotype switch the B lymphocytes
so that most produce IgA. Following exposure to antigen
T suppressor inducer cells (T_{si}) within the gut
lymphoid tissue migrate to the spleen and interact with
certain splenic cells to produce effector T suppressor
cells (T_{sup}). T_{sup} inhibit the production of IgG and
IgM from the respective plasma cells (BG and BM)
resulting in systemic tolerance. T_{si} within the Peyer's
Patches prevent terminal maturation of the lymphocytes
within the lymphoid follicles.
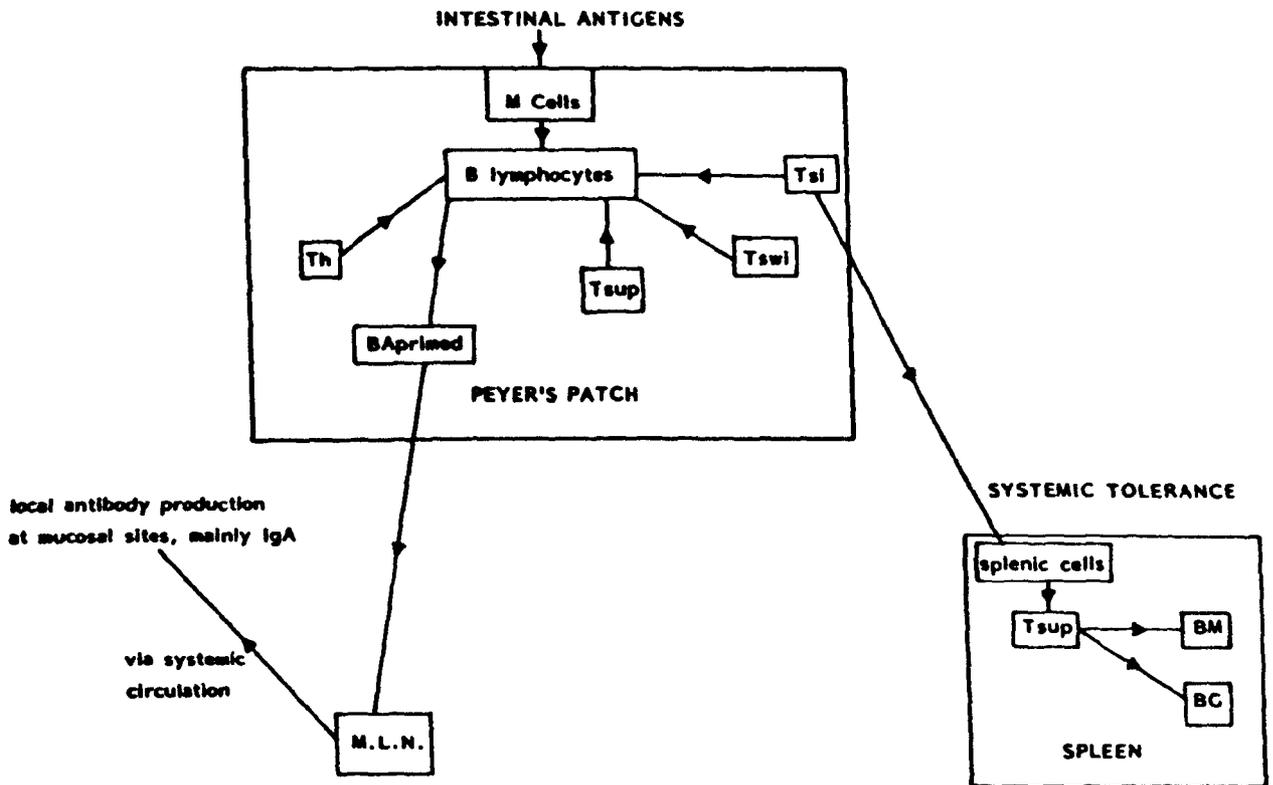
INTESTINAL ANTIGENS

M Cells

B lymphocytes

Tsi

Th

Tsup

Tswi

BAprimed

PEYER'S PATCH

local antibody production
at mucosal sites, mainly IgA

via systemic
circulation

M.L.N.

SYSTEMIC TOLERANCE

splenic cells

Tsup

BM

BC

SPLEEN

Figure 3.1. A simplified flow diagram illustrating the production of systemic tolerance at the same time as local antibody production.

the lymphoid tissue and can migrate to the spleen after
exposure to antigen. In the spleen the Tsi cells
interact with specific splenic cells to produce an
effector T suppressor cell (Tsup) (137). The
simultaneous production of IgA T helper and IgM and IgG
T suppressor cells has been demonstrated in Peyer's
Patches cells in vitro (138) and in vivo (139). Such
results help to explain how a very good local antibody
response can occur at the site of penetration at the
same time that suppressor cells are being induced. The
Tsi cells have no suppressive capacity on their own in
the absence of splenic cells. However, this is by no
means the complete picture; suppression has been
ascribed by some workers to soluble serum factors
(140,141) and there have been reports of distinct T
cell derived soluble suppressors (142). It has been
reported that suppressor cells disappear from the
spleen and the thymus after cell transfer experiments
well before the disappearance of tolerance (143). The
tolerogenic effect of ovalbumin has been investigated
in mice. On absorption across the gut the ovalbumin is
subtly altered into a tolerogenic form which is likely
to exert its tolerogenic effect by activating
suppressor T cells (144,145).

The simultaneous production of secretory antibodies
and systemic tolerance (146), or enhanced antigen

exclusion at the same time as systemic tolerance (147) has been observed. It has also been shown that oral immunization with bacteria may give rise to salivary antibodies and systemic priming rather than tolerance (148). The induction of tolerance to orally administered antigen has been shown to be time and dose dependent (149).

Serum responses to orally administered soluble antigens have been reported in some species (150,151), though no serum antibody response was reported in mice after oral administration of ovalbumin (146). However, a serum antibody response to ovalbumin in mice was reported following a different feeding regimen (149). A small serum response to a single gastric intubation of bovine serum albumin has been reported in Osborn Mendel rats (28) and a small serum response to both repeated gastric and repeated oral administration has been reported in Wistar rats (152,153). The differing responses of different species to an identical feeding regimen has been highlighted in one study (154). All species studied showed similiar levels of circulating antigen, but infants and rabbits showed a serum immune response to BSA which was dose dependent, whilst rats and mice displayed systemic tolerance (154). The work quoted on serum responses to oral administration was carried out in unprimed animals, it is important to

note that the prior immunologic status of the animal

determines the effect of subsequent feeding. If a

soluble antigen is first administered parenterally,

subsequent feedings either fail to induce tolerance, or

may actually boost antibody titers, this depends on the

site of injection (155).

It seems that gut immune responses are neither wholly

negative nor wholly positive, but are the result of

delicate balances between opposing forces. No general

rules apply which allow the accurate prediction of the

outcome of oral administration. Though it seems likely

that the site of antigen uptake in the gut will

influence to a great extent the response induced, if

any. Physiologically, it is likely that the main role

of intestinal IgA is to reduce the absorption of

antigens across the gut, that is immune exclusion. The

role of systemic tolerance is likely to be defensive

also, it may act to prevent the development of certain

hypersensitivity and auto-immune diseases by preventing

damaging immune reactions to antigens that have escaped

immune exclusion.

The first objective relating to animal work in this

study was to generate a significant positive salivary

immune response to a soluble antigen and to demonstrate

that this could be detected by an ELISA. It has been

reported that salivary immune responses to DNP

haptenated species are relatively easy to induce by salivary gland vicinity (SGV) injection alone, or by a combination of mucosal site injection and oral administration (157-159). Therefore, two protein carriers, MYO and BGG, were haptenated with DNP and used as immunising agents in experiments involving SGV injection. However, most studies that have successfully induced a salivary response have used long term feeding of antigen or non-viable bacteria. Salivary responses have been shown to be generally short lived (130,131,148), suggesting that after migration of antigen sensitive cells from Peyer's Patches to salivary glands, cells do not continue to secrete antibodies in the absence of any local challenge. It has been demonstrated that a reproducible salivary immune response is difficult to achieve and depends to a great extent on the immunization protocol used (148). It was shown that repeated gastric intubation (g.i.) of an antigen over at least three days is required to induce a reproducible and significant positive salivary immune response in mice (148). The response to repeated g.i. has been confirmed in the rat. It has also been shown that a much better salivary response is achieved if a booster dose of antigen is administered by g.i. more than two months after the original exposure (25).

The ELISA technique had been validated for the determination of OVA-specific rat IgA and IgG antibodies in the sera following i.p. injection (chapter 2). We now hoped to extend this work to the determination of anti-OVA IgA and IgG antibodies in rat saliva. Therefore, a series of immunisation protocols was undertaken in the attempt to induce a significant positive salivary immune response. After the establishment of a reliable immunisation protocol, we could then attempt to enhance the salivary response by association of the model protein with the chosen colloidal carriers.

In all experiments, involving g.i. of antigen, saliva was collected two weeks after the first dose of the daily repeated gastric intubation regime. Since salivary antibody levels are generally accepted as peaking at about this time (95,145,159).

3.2. Materials and apparatus.

Male Wistar rats 200g were used throughout.

Pentobarbitone sodium 60mg/ml - 'Sagatal' M & B
veterinary, Dagenham, Kent.

Halothane - ICI plc, Macclesfield, Cheshire.

Pilocarpine 1% eye drops - Kirby Warrick, Mildenhall,
Suffolk.

Horse heart myoglobin type III (MYO) - Sigma chemicals,
Poole, Dorset.

Bovine gamma globulin fraction II (BGG) - Sigma.

Ovalbumin grade III - Sigma.

Freunds adjuvant, complete - Sigma.

2,4,-Dinitrobenzene sulphonate (DNP) - K & K labs,
Hollywood, California.

Sodium carbonate - BDH chemicals, Poole, Dorset.

Micro-haematocrit centrifuge - Hawksley and Sons Ltd,
Lancing, Sussex.

Micro-haematocrit capillary tubes, plain and
heparinised - Hawksley and Sons Ltd.

Micro eppendorf tubes, 500μl - Sarstedt, West Germany.

Oral dosing needles - made from a curved syringe needle
(size 19g x 2") with a smoothed rounded piece of silver
solder on the end.

Anaesthetic apparatus - Fluotec, Cyprane Ltd.,
Keighley.

3.3. Methods.

3.3.1. Gastric intubation.

The solution was administered into the stomach
via a dosing needle inserted down the oesophagus.

3.3.2. Saliva collection.

Animals were anaesthetized with i.p.
pentobarbitone 0.1ml/100g or by inhalation of
halothane 2%. Saliva was stimulated by the i.p.
injection of pilocarpine 0.1ml/100g, 1mg/ml and was
collected into plain capillary tubes from the corner of
the mouth as the animal was laid on its back, slightly
tilted to the collection side.

3.3.3. Sera collection.

In earlier terminal experiments, blood samples
were taken by cardiac puncture from pentobarbitone
anaesthetized animals. However, in later work, when
recovery experiments were performed, blood was
collected by serial sampling from the tailtip into
heparinised capillary tubes from halothane
anaesthetized animals. The capillary tubes were then

sealed in a bunsen flame and the sera was collected by spinning down the blood in a capillary centrifuge.

3.3.4. 2,4,-Dinitrophenol haptenation.

One gram of the protein to be haptenated and 1g of potassium carbonate were dissolved in 50ml of water, 1g of dinitrobenzene sulphonate was added and the mixture was stirred gently at room temperature for 18 hours in a conical flask surrounded by aluminium foil. The mixture was then dialysed against running tap water at 4°C for 48 hours. Dialysis was then continued against distilled water for two days with six changes of water (method supplied by Beechams).

3.3.5. Immunisation protocols.

3.3.5.1. Salivary gland vicinity injections.

(i). 2 x 1ml of 0.5ml MYO/DNP 500µg/ml and 0.5ml FCA was injected subcutaneously at the sites of the salivary glands in four rats, saliva was collected ten days later.

(ii). 4 x 0.2ml of 0.1ml BGG/DNP 2mg/ml and 0.1ml FCA was injected subcutaneously at each of the four main salivary glands, saliva was collected ten days later.

3.3.5.2. Salivary gland vicinity injection and gastric intubation.

Initially, 200μg BGG/DNP was gastrically intubated in 200μl PBS into six rats. Ten days later, 200μg BGG/DNP in 200μl FCA was injected subcutaneously into each rat at the salivary glands. Seven days later, the SGV injection was repeated, saliva was collected five days later.

3.3.5.3. Repeated gastric intubation of myoglobin.

(i). 200μg MYO in 200μl PBS was intubated gastrically on four consecutive days to six rats, saliva was collected fourteen days after the initial intubation.

(ii). 200μg MYO in 200μl PBS was intubated gastrically on four consecutive days to six rats. The animals were then left untreated for more than two months. A further dose of 200μg MYO in 200μl PBS was then intubated gastrically, saliva was collected two days later. This long term boosted response is termed the "memory" response.

3.3.5.4. Repeated gastric intubation of ovalbumin.

One milligram of ovalbumin in 250μl PBS was gastrically intubated on four consecutive days to six rats, saliva was collected fourteen days after the initial intubation.

For all immunisation protocols, the group mean ELISA
saliva results from the immunised animals was compared,
in an Unpaired Students "t" test, with the results for
blank saliva collected from animals which had received
identical treatment, but the immunising antigen had
been omitted. All ELISA values are quoted as optical
density readings at 405nm; only the values from the
lowest dilutions of saliva assayed are shown. The group
mean values are considered to be statistically
significant if in the "t" test, $p < 0.05$, an *
indicates when this is so.

3.4. Results.

3.4.1. Salivary gland vicinity injections.

Detectable salivary immune responses could not be
detected from any of the animals immunised by SGV
injection alone, or by combined g.i. and SGV of
BGG/DNP. None of the values obtained from the immunised
animals differed from the values obtained from the
blank groups. Table 3.1. shows the results from a
typical assay for IgA in saliva. Many other similiar
assays were performed, with slightly different assay
conditions in each case, but all proved negative for
both IgA and IgG. Since in all cases these results were
negative they have not been shown. Because of the

failure of these immunisation protocols, in subsequent experiments, the model antigens were administered by repeated g.i.

3.4.2. Repeated gastric intubation of myoglobin.

The salivary immune response to repeated gastric intubation of myoglobin is shown in table 3.2. This table also shows the salivary memory response to myoglobin, though these results were obtained from a different group of experimental animals.

The repeated g.i. of MYO at 200µg per day for four days did not induce a significant positive salivary response. However, when a booster dose of MYO was given by g.i. more than two months later, a significant positive salivary IgA response was elicited (significant at $p < 0.01$ when compared with 'blank' saliva in Unpaired Student "t" test).

3.4.3. Repeated gastric intubation of ovalbumin.

The salivary immune response to the repeated gastric intubation of ovalbumin is shown in table 3.3. The repeated g.i. of OVA at 1mg per day for four days induced a significant positive salivary IgA response (significant at $p < 0.02$ when compared with 'blank' saliva in Unpaired Student "t" test).

Table 3.1. ELISA saliva response following gastric
intubation, followed by repeated salivary gland
vicinity injection of BGG/DNP (mean ±SD n=6).

	IgA
Blank group	0.130 ±0.010
Immunised group	0.133 ±0.005

Saliva samples diluted 1/5 in PBS.

Table 3.2. ELISA salivary response following repeated
gastric intubation of myoglobin (mean ±SD n=6).

	IgA	IgG
Blank group	0.490 ±0.087	0.786 ±0.055
200μg x 4 repeated	0.539 ±0.047	0.704 ±0.104
Memory saliva response.		
Blank group	0.675 ±0.100	0.327 ±0.010
Memory group	1.429 ±0.427 *	0.388 ±0.048

All saliva samples diluted 1/5 in 1%BSA/PBS.

Table 3.3. ELISA salivary response following repeated
gastric intubation of ovalbumin (mean ±SD n=6).

	IgA	IgG
Blank saliva	0.615	0.335
	0.640	0.311
1mg x 4 repeated	0.969 ±0.124 *	0.322 ±0.009

Saliva samples diluted 1/10 in 1%BSA/PBS, only two
blank samples were run in this assay.

3.6. Discussion.

The objective of this work was to establish an immunisation protocol which would induce a significant positive salivary immune response in the Wistar rat. Salivary gland vicinity injections alone, or in combination with gastric intubation, with a DNP-haptenated protein did not generate a significant positive salivary antibody response. Most researchers have reported that salivary immune responses are generally very difficult to achieve and this perhaps is not particularly surprising since the normal physiological response following absorption of antigen across the gut is likely to be tolerance. A failure in this protective mechanism may lead to damaging consequences for the individual. Challacombe has shown that in mice, repeated oral administration of antigen over at least three days is required (148). He has also reported that SGV injections are not a very reliable method of inducing a positive salivary immune response in rats (personal communication). The work published supporting the use of SGV did not offer any explanation as to why this route of administration of antigen should be any better than a simple sub-cutaneous injection of antigen. In retrospect, it must be concluded that SGV injection was not a very good

immunisation protocol to select. Also, the ELISA

technique for the determination of DNP-specific

antibodies was hampered by the release of unreacted DNP

from the conjugate in solution. This problem is likely

to result in reduced sensitivity of the assay, since

DNP-specific antibodies may bind to a group that is

subsequently lost into solution as the assay

progresses. Therefore, it was obvious that the dialysis

technique described in section 3.3.4., was not

effective at removing all unreacted DNP from the

conjugate. This problem could have been resolved by

removing the unreacted DNP by column chromatography,

but this did not prove necessary as the DNP-haptenated

species were replaced by native protein models for oral

administration studies. The use of native protein

antigens allowed us to demonstrate that there was no

major structural or conformational changes in the

protein during its incorporation into, or adsorption

onto, the colloidal carriers (see section 2.3.). If the

DNP-haptenated species was used, the proteins may have

been extensively degraded, but we may still detect

immune responses to the DNP hapten on protein

fragments. We are interested in immune responses to an

intact protein following its oral administration.

Significant positive salivary immune responses were

induced following gastric intubation alone. The

boosted, or "memory" immune response was considerably greater than the primary immune response, with myoglobin, but this response was generated after a delay of more than two months. This made the experiment very time consuming and expensive, since the experimental animals needed to be kept over an extended period.

Positive immune responses were detected in saliva that was not processed in any way prior to freezing. There had been some worry that the various substances present in saliva, mucins, food, enzymes etc. might inactivate or denature the antibodies, but this did not prove to be so. Although, there is a possibility that the detected responses may have been considerably greater if some processing step eg. enzyme inhibition, or removal of particulates, had been undertaken.

It was decided that the results obtained from the immunisation protocols undertaken justified moving onto the next stage of the project, the incorporation of ovalbumin into colloidal carriers and the assessment of the immune responses following gastric intubation of these carriers.

An immunisation protocol involving the repeated gastric intubation of 1mg ovalbumin per day, for four days, was selected to be used in subsequent studies. In later work, when recovery experiments were allowed, the

memory response to gastrically intubated ovalbumin was also assessed.

4. LIPOSOMES.

4.1. Introduction.

A number of studies has investigated the potential of liposomes as delivery systems for the oral administration of macromolecules, particularly insulin (19-23). Although some of this work met with a modicum of success, liposomes are considered as unsuitable for the oral delivery of active macromolecules in humans since only limited and variable absorption has been achieved to date. However, limited uptake of macromolecules may lead to the induction of immune responses and liposomes may prove effective candidates for oral immunisation studies.

Liposomes which are claimed to be stable in the g.i.t. can be easily prepared. Distearoylphosphatidylcholine/cholesterol 1/1 ratio (DSPC/CHOL) liposomes are reportedly stable at low pH, in the presence of 10mM bile salts and at 37°C in the presence of pancreatic lipase (165). However, the reported stability of DSPC/CHOL liposomes in the g.i.t. has recently been brought into question following work involving the incubation of liposomes in simulated intestinal media (166).

A carrier which protects entrapped material from degradation in the g.i.t. and encourages uptake via the

PP is likely to prove an effective adjuvant in oral immunization studies.

There has been considerable controversy concerning the reported absorption of intact liposomes across the g.i.t. In 1981, Diwaker et al (167) concluded that they were not absorbed intact, but they could still promote the intestinal absorption of an entrapped macromolecule by protecting it from rapid digestion. More recently the intracellular digestion of liposomes by mucosal cells has been reported in a vascularly perfused rabbit ileum model in vitro, with transport of an entrapped marker into the venous effluent as free molecules (168). However, a more recent study, concerning both in vitro and in situ data, has confirmed that liposomes are not taken up by epithelial cells and that liposomal entrapment does not facilitate the transport of non-absorbable drugs, and prevents the transport of absorbable ones (166).

It had been previously reported, however, that gut epithelial cells take up DSPC/CHOL liposomes intact by adsorptive endocytosis since the liposomes will be fairly 'solid' at 37°C due to the high transition temperature of DSPC (169,170).

If intact liposomes were to reach the serosal side of the intestine ,they could then enter either the lymphatic system or the portal blood. One study has

reported that the majority of the absorbed material
enters the lymph (21), this would make an immune
response more likely, since the liposomal material
would rapidly encounter lymphoid tissue in the draining
lymph nodes. If the liposomes were to be delivered to
the general circulation, their fate would then depend
on factors such as size, surface charge, opsonisation
and phospholipid composition. Interestingly, DSPC/CHOL
liposomes are one of the most stable compositions when
examined in in vitro experiments with plasma (171).

The potential of liposomes as immunological
adjuvants was recognised early (171) and there is now
much literature relating to this. It appears that the
immunogenicity of most proteins can be enhanced by
association with liposomes, though no single ideal
formulation has arisen. It seems that protein does not
need to be incorporated into the liposome, since
liposomes have served as adjuvants for a small amount
of materials adsorbed on the surface, when no
enhancement was seen for completely encapsulated
material (173,174). Some success has been achieved with
liposomes as oral adjuvants. In several studies,
liposomes have been shown to be effective at inducing
salivary IgA antibodies against various Streptococcus
mutans fractions (25-27). Oral administration of BSA in
liposomes can also lead to an enhanced salivary IgA

response (28). In none of these studies were the
liposomes claimed to be stable in the g.i.t.

The method chosen for the preparation of liposomes
in this study is one capable of encapsulating a wide
variety of materials, with high efficiency, under mild
conditions into vesicles of variable lipid composition
(175,176). Multilamellar vesicles were prepared, since
these afford maximum protection against the effects of
enzymes, bile salts and extremes of pH in the gut
(175,176).

4.2. Materials and apparatus.

Distearoylphosphatidylcholine - Sigma Chemical

Co.,Poole, Dorset, U.K.

Cholesterol - Sigma 99+% grade.

Ovalbumin - Sigma Grade III.

Sonic probe - Dawe 7532B, Branson sonic power Co, England.

Rotary evaporator - Buchi, Rotavapor-R, Flawil, Switzerland.

Freeze drier - Edwards modulyo, Crawley, Sussex.

Malvern 2600D laser sizer - Malvern Instruments, Malvern.

4.3. Methods.

Four batches of liposomes were prepared by the method described below. Aliquots were removed for determination of particle size, by laser diffractometry, and for determination of ovalbumin entrapment, by Lowry assay. The liposomal suspensions were then adjusted to the required volume for gastric intubation according to the protocol described.

4.3.1. Liposome preparation.

DSPC 26mg and CHOL 12.8mg were deposited as a thin lipidic film on the walls of a 50ml quickfit flask

-117-

under rotary evaporation from chloroform. The film was dried with nitrogen before being resuspended by vigorous shaking at 40°C with 2ml water. The preparation was left to stand for 30 minutes to allow full rehydration of the lipids and then mixed with 2ml (100mg/ml) ovalbumin solution and freeze dried.

Rehydration of the liposomes was achieved by addition of 200ul of water at 40°C. The liposomes were kept at this temperature for 30 minutes before being diluted with 30ml PBS. The liposomes were collected by centrifugation at 3000 rpm for 30 minutes and free ovalbumin was removed by repeated washing in PBS.

4.3.2. Ovalbumin entrapment.

A 1ml aliquot of the liposomal suspension was sonicated to disrupt the liposomal membranes and the protein content was assessed by a Lowry assay (see section 2.5.3.).

4.3.3. Particle size analysis.

A small aliquot of the liposomal suspension was removed and sized on the Malvern 2600D laser sizer according to the method published in the technical literature. A description of the technique and a diagrammatic representation of the apparatus are given in section 6.3.3.2. and figure 6.2.

4.3.4. Immunisation protocol.

Male Wistar rats of about 200g were used, divided into three groups of eight animals;

Group A received 1mg ovalbumin,

Group B received blank liposomes,

Group C received 1mg ovalbumin encapsulated in liposomes.

Each dose was given by gastric intubation in 0.4ml PBS and each dose for each group of animals was repeated over four consecutive days. Sera and saliva samples were collected two weeks after the initial intubation, diluted in 1% BSA in PBS and stored frozen at -20°C until they were assayed by ELISA. All subsequent saliva and sera samples were treated in the same way after collection.

4.4. Results.

4.4.1. Ovalbumin entrapment.

The mean ovalbumin entrapment in the liposomes was 8.88mg, which was 4.4% of that added initially. The results for each individual batch of liposomes used for oral intubation in group C are shown in table 4.1.

4.4.2. Particle size analysis.

The results from the particle size analyses on the same four batches of liposomes described above are shown in table 4.2. The mean particle sizes of the different batches were 5.79, 5.25, 5.77 and 5.22μm respectively.

4.4.3. Enzyme linked immunosorbent assay.

The ELISA results are shown in tables 4.3. and 4.4. Values are quoted as optical density readings at 405nm, for 1/10 dilutions of both sera and saliva. A reference wavelength of 690nm was included for the sera determination, since this measurement was performed in a dual wavelength spectrophotometer which was on short term loan to the laboratory. The reference wavelength reading was subtracted from the reading at 405nm to give the values shown in table 4.3. (sera only). The subtraction of this reference value precludes possible false positive results in the assay due to anomalies in the plastic of the microtitre plate, but this was considered an unnecessary precaution and was not subsequently used.

None of the group responses were statistically significantly different in an Unpaired Students "t" test.

Table 4.1. Ovalbumin entrapment in liposomes as determined by Lowry assay (mean ±SD n=4).

Liposome batch	Ovalbumin entrapment(mg)	Ovalbumin entrapment(%)
1.	9.4	4.7
2.	8.75	4.4
3.	8.75	4.4
4.	8.6	4.3
Mean ±SD	8.88 ±0.357	4.4 ±0.173

Table 4.2. Size analysis of liposomes by laser

diffractometry (n = 4).

Batch	1.	2.	3.	4.
size μm	weight % undersize			
118.4	100	100	100	100
54.9	99.8	100	100	100
33.7	98.5	99.7	100	100
23.7	97.3	98.6	99.9	99.8
17.7	93.5	95.2	98.4	97.9
13.6	85.9	89.2	91.7	91.5
10.5	80.6	83.6	83.1	87.0
8.2	73.9	76.6	73.3	80.9
6.4	56.6	62.8	56.9	64.5
5.0	40.7	46.7	40.8	46.8
3.9	30.6	33.5	29.1	34.3
3.0	16.2	18.5	16.2	18.2
2.4	3.6	5.6	4.7	4.6
1.9	0.7	1.3	1.0	1.3
1.5	0.2	0.4	0.3	0.7
1.2	0.1	0.2	0.2	0.4
mean size μm	5.79	5.25	5.77	5.22

Table 4.3. ELISA saliva and sera responses to gastric intubation of ovalbumin in liposomes (mean ±SD n=8)

(i). Saliva.

	IgA	IgG
Group A	0.473 ±0.116	0.427 ±0.028
Group B	0.453 ±0.092	0.407 ±0.027
Group C	0.540 ±0.180	0.434 ±0.028

(ii). Sera.

	IgA	IgG
Group A	0.061 ±0.021	0.154 ±0.049
Group B	0.059 ±0.017	0.160 ±0.035
Group C	0.066 ±0.017	0.189 ±0.033

4.5. Discussion.

Although the entrapment efficiency of ovalbumin in the liposomes was low, 4.4% of 200mg added initially, it was sufficiently reproducible and efficient to allow the intubation of ovalbumin at the required dose of 1mg per animal per day, from one batch of liposomes prepared per day. The entrapment value was comparable with a value published in the literature for the entrapment of albumin in the same type of multilamellar vesicles prepared in the same way (albumin at 10mg/ml entrapment efficiency of 3.1%) (175).

The liposomes were heterogeneous in size, but the mean size was quite reproducible for the different batches. It was thought that much of the material at the upper end of the size distribution was lipid aggregates and not liposomes with entrapped ovalbumin. The literature reports that for liposomes prepared by this method and sized under an electron microscope, 95% of liposomes are <1μm in size after the removal by centrifugation of large lipid aggregates (175). The removal of large lipid aggregates was not considered necessary for our purposes, since the liposomes were to be administered orally. Therefore, the actual mean particle size of the liposomal material was probably smaller than that shown in table 4.2.

The ELISA results indicated that entrapment of ovalbumin in DSPC/CHOL liposomes led to slightly greater immune responses following gastric administration alone. Both sera and salivary responses were slightly greater in the liposomal group, with the IgA response more affected in the saliva and IgG more so in the sera. However, none of the liposomal group responses were statistically significantly greater than the equivalent responses from the control group.

The slightly greater sera responses from the liposomal group indicates that there may possibly be enhanced transport of liposomally entrapped material across the epithelial cells of the gut and passage into the blood or lymph. Whether enhanced transport may be due to the uptake of intact liposomes, the enhanced uptake of entrapped material alone, the protection from degradation of entrapped material, or perhaps even a combination of one or more of these mechanisms, is not known. The slightly enhanced salivary responses from the liposomal group may indicate that DSPC/CHOL liposome entrapped material gains better access to the tissue of the PP than soluble material. However, since none of the immune responses for the liposomal group were significantly greater than control responses, the ELISA results may be better explained by reference to the reported lability of DSPC/CHOL liposomes in

simulated intestinal media and the reported lack of
absorption of entrapped markers by intestinal cells
(166). Therefore, it was decided that the next
experiment would be performed with polyacrylamide
microparticles (PA), these seemed to have greater
potential as possible oral adjuvants, for reasons
discussed in chapter 5.

A major cause of concern was the failure of the
control group to produce a significant positive
salivary immune response, relative to the blank group,
even though the immunisation protocol was identical to
that in 3.4.3., when a significant positive salivary
immune response was induced. However, the 'blank' group
in this experiment had received gastric administration
of liposomes. It is possible, although unlikely, that
this produced a non-specific enhancement of the
salivary immune response such that the background
"blank' response was raised. These results bring into
question the statistical significance of the salivary
immune response achieved in 3.4.3., particularly as
this was assessed with reference to only two blank
animals.

Since a significant positive salivary immune
response was not reproduced in this experiment, it was
decided that primed animals would be used in the next
experiment. We had hoped to use oral administration

only at this stage, since this has a number of
advantages over parenteral, or combined therapy, when
considering human subjects. It was of interest to see
how an oral prime might affect the sera response to
gastrically administered antigen. Therefore, the
animals in experiments PA1 and PA2 (see chapter 5 for
description) received a priming dose of 20mg ovalbumin
by gastric intubation. It has been shown that this dose
administered by gastric intubation is ineffective at
producing a detectable salivary antibody response in
mice (148). Therefore, it might prove an effective
priming dose. However, a different response may be
induced in rats. The animals were primed two weeks
prior to the commencement of the repeated gastric
intubation regime.

Oral priming with cholera toxin has been shown to
be superior to parenteral inoculation in generating a
specific secretory IgA response (95,155), with the
exception of intraperitoneal priming (155).
Combinations of crude toxin and toxoid were most
effective and a single dose of this combination induced
mucosal priming which was fully developed in two weeks
(155). The best cellular immune response to cholera
toxin is obtained when a single i.p. injection is
followed two weeks later with an intestinal boost (25).
The effectiveness of an i.p. prime followed by an oral
boost for producing an effective secretory immune

response has been confirmed using ovalbumin in sheep

(159).

5. POLYACRYLAMIDE MICROPARTICLES.

5.1. Introduction.

The preparation of polyacrylamide microparticles with an entrapped macromolecule was first reported in 1975 (177); with a detailed production procedure being published the following year (178). The method was subsequently altered to produce more spherical particles of a narrower size distribution (179).

Since this early work, the approach has been extensively modified to produce more biodegradable particles. For example the polysaccharides dextran and starch are first derivatized with acrylic acid glycidyl ester and the resulting acrylic polysaccharides are then polymerised (180,181). Macromolecules entrapped within these polyacryldextran or polyacrylstarch microparticles display the same general properties as those entrapped in the original polyacrylamide microparticles (180,181).

Polyacrylamide microparticles appear to have considerable potential as orally administered immunological adjuvants, in particular for the stimulation of the secretory immune response. Particles of this type have been shown to be effective immunological adjuvants for entrapped macromolecules when administered to experimental animals by other

routes (182-184). Polyacrylamide microparticles are an effective adjuvant for entrapped L-asparaginase when administered by the intraperitoneal or the intravenous route to mice. However, only a weak immune response to this enzyme was induced when it was administered entrapped in microparticles by the subcutaneous or the intramuscular route (184). This indicates that the microparticles are not effective adjuvants for an entrapped macromolecule for all routes of administration.

The adjuvant properties of this type of acrylic microparticle has been confirmed using other entrapped macromolecules. In one experiment, human serum albumin, entrapped in microparticles and in the soluble antigen form, were administered intravenously to mice. The microparticles induced a strong antibody response, whilst for the soluble form no response was seen (182). Simil ar results were obtained when the particulate formulation was administered by the intramuscular route. The antibody response obtained was similiar to that produced with Freund's complete adjuvant (183).

It was concluded from this work that the microparticles functioned as a pure adjuvant, amplifying both the cellular and the humoral immune responses when administered by certain routes (185).

The adjuvant effect is thought to be due to the macroporous structure of the microparticles in which the entrapped macromolecule is exposed to the environment at the surface (181,186,187). The antigenic determinants of the entrapped macromolecule are in this way exposed to the immunocompetent cells in an immobilised form at a high concentration. In addition, the microparticles have been shown in vitro to be readily taken up by macrophages (188,189). The macrophages are possibly the most important cells involved in presenting antigens to the T helper cells, and are responsible for the processing of both soluble and particulate antigens. Particulate antigens are internalized and processed, after which they are presented on the macrophage surface and are then recognised by T cells (190). Thus macrophages are important in the effective stimulation of a T cell dependent immune response.

Experimental evidence indicates that entrapment of a macromolecule within the microparticles does not seem to result in major changes of conformation of the macromolecule. This was demonstrated when no auto-antibody response could be detected to mouse serum albumin when it was administered entrapped in microparticles to experimental mice (182). Enzymes entrapped within these microparticles retain much of

their enzymatic activity (178-181,184,187,191). This observation provides further evidence that entrapment is a "mild" procedure.

It has been demonstrated that the humoral immune response to an entrapped antigen can be assessed using standard immunoassay techniques (182-184).

It has been shown that entrapment of a macromolecule in polyacrylamide microparticles confers enhanced stability on that macromolecule against both proteolytic and heat denaturation (178-181). Entrapment of the enzyme, carbonic anhydrase, led to enhanced protection against degradation by protease VI, a proteolytic enzyme with broad activity (179). Furthermore, it has been shown that acidic pH (>1.2) does not adversely affect the microparticle matrix (187). Therefore, the entrapment of macromolecules within these microparticles may lead to enhanced protection for the macromolecule against the degradative properties of gastric acidity and the gastrointestinal enzymes.

Although the material exposed at the surface of the particle is likely to be extensively degraded in the normal way, material more centrally located in the particle may be better protected. An increase in the amount of the macromolecule absorbed may lead to a modification of the immune response produced,

particularly if the entrapped material is only released from the particles slowly. Polyacrylamide microparticles are only slowly eliminated in vivo, they have a half life of 10-15 weeks in the liver and the spleen (191). Therefore, it is likely that if the particles are taken up by the PP, they will remain at this site for some time. It has been suggested that most particles taken up by the PP will be sequestrated into macrophages and there is little evidence for significant transport of particles to other sites in the body (71).

Results have shown that there is considerable leakage of ovalbumin from polyacrylamide microparticles on storage in vitro (178,179). Since the particle matrix is only slowly degraded in vivo, it is likely that there wil be continued release of entrapped material after uptake, if indeed they are taken up. The effect of this on the immune response produced is not known, but an enhanced or longer sustained response might be expected.

It has been repeatedly demonstrated that polyacrylamide microparticles, of the required size characteristics, with an entrapped macromolecule can be prepared (179,184,188,189,191).

Microparticles were prepared by the emulsion polymerisation of acrylamide and bisacrylamide in the

presence of the protein to be entrapped. Before the

microparticles were administered to experimental

animals, the extent of leakage of the entrapped protein

was determined.

5.2. Materials and apparatus.

Acrylamide, bisacrylamide and ammonium persulphate - Bio-rad electrophoresis purity reagents, Richmond, California, U.S.A.

Ovalbumin - grade III, Sigma chemicals, Poole, England.

TEMED (N,N,N',N'-tetramethylethylenediamine) - Sigma.

Pluronic F68 - Wyandotte chemical corp., Wyandotte, Michigan, U.S.A.

Ultraturrax TP 18-10 - Janke and Kenkel KG, Staufen, Breisgau, W.Germany.

Coulter counter model TA - Coulter Electronics Ltd, Harpenden, Hertfordshire, England.

30μm aperture tube - Coulter Electronics.

Isoton - Coulter Electronics.

Polybead polystyrene microspheres (2.88μm) - Polysciences Inc. Warrington.

5.3. Methods.

5.3.1. Microparticle preparation.

100mg ovalbumin, together with 475mg acrylamide and 25mg bisacrylamide, were dissolved in 5ml 0.2M sodium phosphate buffer pH 7.4 and (1mM) EDTA and the mixture was deoxygenated in a bell jar connected to a vacuum pump.

The catalyst, 200µl ammonium persulphate 0.43M (adjusted to pH 7.4 with NaOH) was added and the aqueous phase was homogenised together with 100mg Pluronic F68 in 300ml organic solvent consisting of chloroform-toluene (1:4). The organic phase had previously been deoxygenated by bubbling nitrogen gas through for at least 15 minutes, deoxygenation was continued during the polymerisation process.

Homogenisation achieved the production of a water-in-oil emulsion and polymerisation was then initiated by the addition of 250 µl of the initiator N,N,N',N'-tetramethylenediamine.

After preparation, the particles were allowed to sediment over a 15 minute period and the upper organic phase was then decanted off. The particles were collected by centrifugation at 2500 rpm for 1 hour, resuspended and washed repeatedly in buffer before finally being resuspended in saline.

The microparticle composition was characterised by the T-C nomenclature suggested by Hjerten (192). The T indicates the total amount of monomer used (g per 100ml solvent) and the C indicates the % of bisacrylamide used (w/w) of the total monomers. The particles prepared had a T-C of 10-5; this composition gives a good entrapment efficiency for proteins whilst not causing denaturation. High concentrations of monomer

have been shown to cause denaturation of proteins (193).

5.3.2. Ovalbumin entrapment.

The microparticles were fully resuspended with a pasteur pipette and a 25ml syringe and an aliquot was removed for amino acid analysis by the method described in section 2.6.

5.3.3. Ovalbumin leakage.

The microparticles were stored for three months at 4°C prior to their use. Therefore, it was necessary to determine the extent of leakage of the entrapped protein. An aliquot of the microparticle supernatant was removed from above the sedimented particles and a Lowry assay was performed as described in section 2.5.3.

5.3.4. Particle size analysis.

The 30μm aperture tube was fitted to the Coulter Counter and the apparatus was calibrated with polystyrene microparticles of known size, according to the method described in the Coulter Electronics technical literature. The apparatus was then cleaned thoroughly and the polyacrylamide microparticles to be sized were dispersed in Isoton with a mechanical

stirrer. A size analysis was then performed, according to the method described in the technical literature.

5.3.5. Immunisation protocols.

Older and consequently larger animals were used in the initial experiments with polyacrylamide microparticles (PA1 and PA2) since these would be easier to handle and a greater volume of saliva could be collected. There was also the possibility that the secretory immune system might be better developed in older animals, so they might produce better immune responses.

The dose of ovalbumin gastrically intubated in experiments PA1 and PA2 needed to be reduced below that which had been given to experimental animals previously (in chapters 3 and 4), because of limitations imposed by the amount of material entrapped in the microparticles. In experiments PA1 and PA2, 0.5mg ovalbumin was gastrically intubated, 1mg had been administered in previous experiments.

In experiments PA1 and PA2, animals were killed when saliva was collected. This was necessary because a licence from the Home Office for recovery experiments had not yet been obtained. Consequently a separate experiment was run, PA2, to enable the assessment of the response to a booster dose of ovalbumin entrapped

in microparticles, also administered by gastric
intubation, two weeks after the repeated gastric
intubation regimen.

In experiment PA3, sequential sampling of sera and
saliva was performed for the first time. Therefore, a
full assessment could be made in the same group of
animals of any changes in immune responses to the
gastric intubation of ovalbumin.

(i).   PA1 experiment.

Male Wistar rats of about 300g were used and all
animals received a priming dose of 20mg of ovalbumin in
0.5ml of physiological saline by gastric intubation.

Two weeks later the animals were subdivided into
three groups of eight animals;

Group A received 0.5mg of ovalbumin entrapped in
microparticles,

Group B received blank microparticles without
entrapped ovalbumin,

Group E received 0.5mg of soluble ovalbumin.

All doses were administered in 0.5ml physiological
saline by gastric intubation and each of the
intubations for each group of animals was repeated over
four consecutive days. Sera and saliva samples were

collected two weeks after the commencement of the repeated intubation and stored frozen.

(ii). PA2 experiment.

The first part of this experiment was identical to experiment PA1. Male Wistar rats of about 300g were used and each animal received a priming dose of 20mg ovalbumin by gastric intubation. Two weeks later the rats were divided into two groups of eight animals;

Group C received 0.5mg of soluble ovalbumin,

Group D received 0.5mg of ovalbumin entrapped in polyacrylamide microparticles.

Each dose was administered in 0.5ml physiological saline by gastric intubation and each dose for each group of animals was repeated over four consecutive days.

Two weeks later all the animals in groups C and D received an additional dose of ovalbumin, again by gastric intubation;

Group C received 0.4mg soluble ovalbumin,

Group D received 0.4mg ovalbumin entrapped in polyacrylamide microparticles.

Each dose was administered in 0.5ml physiological saline. Sera and saliva samples were collected one week after this booster dose and stored frozen.

In experiments PA1 and PA2, sera and saliva samples were collected under pentobarbitone, as described in chapter 3 and stored frozen until assayed by ELISA and RIA.

(iii).   PA3 experiment.

Male Wistar rats of about 200g were used and all animals received a priming dose of 5mg ovalbumin in physiological saline by intraperitoneal injection. Prior to this, sera and saliva samples were collected from all the animals. The rats were then subdivided into three groups of eight which received the following treatment two weeks later;

Group A received 1mg of ovalbumin entrapped in microparticles,

Group B received blank microparticles without entrapped ovalbumin,

Group C received 1mg soluble ovalbumin.

All doses were administered in 0.25ml physiological saline by gastric intubation, this volume was easier to intubate than 0.5ml, and each of the intubations for

each group of animals was repeated over four consecutive days. Sera and saliva samples were collected two weeks after the commencement of the gastric intubation and stored frozen.

Two months after the commencement of the initial intubations, all the animals in this experiment received a booster dose of 1mg ovalbumin by gastric intubation in 0.25ml physiological saline. Sera and saliva samples were then collected four days later and stored frozen.

All sera and saliva samples in experiment PA3 were collected from animals anaesthetized by inhalation of 2% Halothane, as described in chapter 3, and stored frozen until assayed by ELISA.

5.4. Results.

5.4.1. Ovalbumin entrapment.

Ovalbumin was included in the initial monomer solution at a concentration of 20mg/ml. 7.4% of this was entrapped in the microparticles during preparation, as determined by automatic amino acid analysis on the hydrolysed particles.

5.4.2. Ovalbumin leakage.

20.6% of the entrapped ovalbumin leaked out of the particles during storage at 4°C for three months, as determined by a Lowry assay on the particle supernatant.

5.4.3. Particle size analysis.

Four repeat determinations were performed on the particles and the mean values obtained were plotted (figure 5.1.). The mean (median) particle size was 2.55μm, with 25% <1.45μm and 75% <4.65μm.

5.4.4. Enzyme linked immunosorbent assay.

The positive control samples, both sera and saliva, included in the ELISA were obtained from an animal which was immunised with 500μg OVA in FCA by i.p. injection, the samples were collected 14 days later.

(i). ELISA results from PA1 and PA2 sera and saliva are shown in table 5.1. The values quoted are optical density readings at 405nm for 1/20 dilutions of sera and 1/5 dilutions of saliva. Only results from assays performed on the same day are compared directly in the table. No standard deviation is included for the

CUMULATIVE
% OVERSIZE.
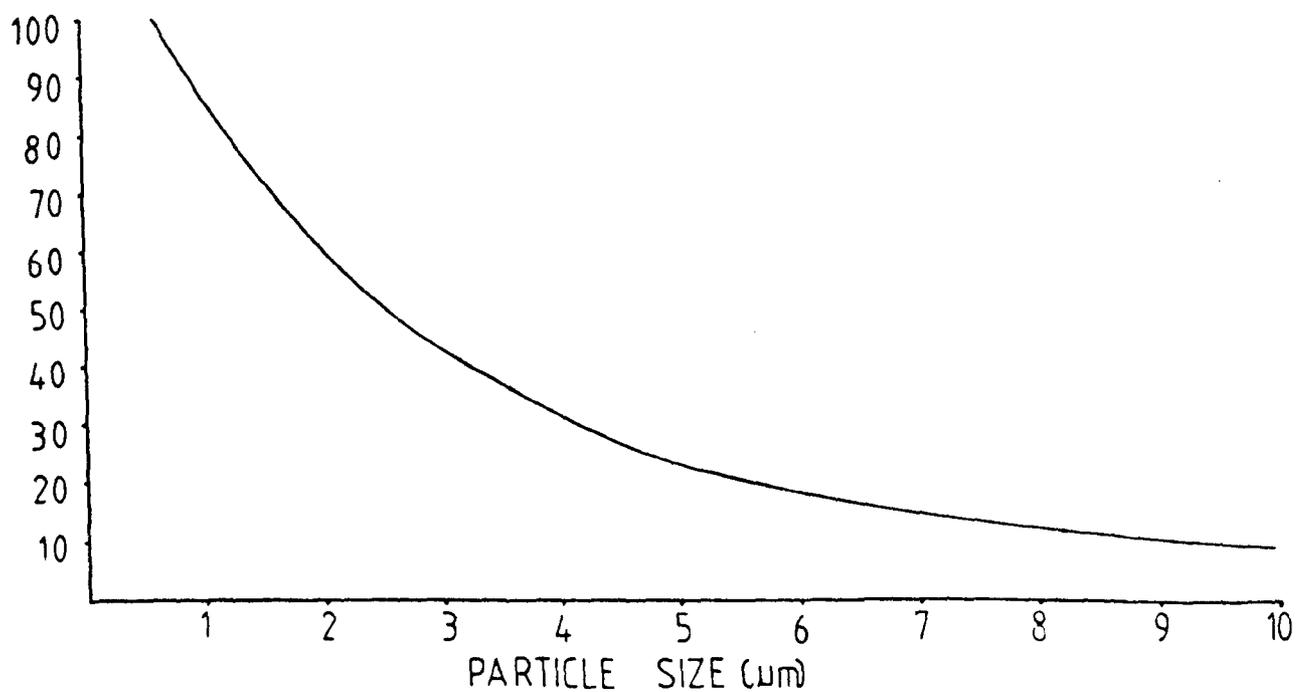
Figure 5.1. Mean particle size of polyacrylamide microparticles determined by Coulter counter (n=4).

positive control sera, because only two determinations were performed on this sample.

Table 5.2. shows the ELISA results from PA1 saliva, groups B and E, which were re-assayed alongside pre-inoculation saliva to determine if the priming dose of ovalbumin had proved to be an effective immunising dose. Values quoted are optical density readings for 1/10 dilutions.

All assay values were low and no significant differences were seen between any of the groups for sera or saliva responses in experiments PA1 and PA2.

(ii). ELISA results from PA3 sera and saliva samples are shown in tables 5.3 and 5.4 respectively. The values quoted are optical density readings for 1/20 dilutions of sera and 1/8 dilutions of saliva. All pre-inoculation (p.i.) samples were pooled prior to assay.

A significant positive salivary IgA memory response was seen in the group of animals receiving g.i. of OVA entrapped in microparticles, group A (significant at $p < 0.01$ in Unpaired Student "t" test when compared with control group).

5.4.5. Radioimmunoassay.

This alternative immunoassay technique (see chapter 2 for method) was performed on the samples from PA1 and PA2 to either confirm the low values generated in the ELISA, or to indicate if the ELISA assay was of relatively low sensitivity. Since the disappointing results obtained had brought into question the sensitivity of the ELISA technique.

The results from RIA of PA1 and PA2 sera and saliva are shown in tables 5.4. and 5.5. respectively, the values quoted are counts per minute for 1/2 dilutions of sera and 1/10 dilutions of saliva, both in PBS.

The positive control included in the RIA was obtained from an animal which was immunised with 10mg OVA in Freunds incomplete adjuvant by i.p. injection, followed by i.p. injection of 4×10^{10} organisms of B.pertussis 10 days later. The serum sample was collected 14 days later.

This assay confirmed the low values obtained in the ELISA and confirmed that there was no significant differences between the immune responses of any of the groups in PA1 and PA2 experiments.

Table 5.1. ELISA results for PA1 and PA2 sera samples (mean ±SD n=8)

PA1 sera

	IgA	IgG
Group A	0.328 ±0.051	0.707 ±0.210
Group B	0.334 ±0.056	0.764 ±0.258
Group E	0.449 ±0.110	0.873 ±0.289
Positive control	1.065	2.830

PA2 sera

	IgA	IgG
Group C	0.338 ±0.049	0.671 ±0.213
Group D	0.263 ±0.032	0.520 ±0.150
Positive control	0.657	1.830

Table 5.2. ELISA results for PA1 and PA2 saliva samples (mean ±SD n=8).

| | PA1 and PA2 saliva | |
	IgA	IgG
Group A	0.253 ±0.112	0.216 ±0.026
Group B	0.231 ±0.068	0.189 ±0.014
Group C	0.232 ±0.080	0.178 ±0.015
Group D	0.265 ±0.080	0.176 ±0.052
Group E	0.292 ±0.138	0.190 ±0.096
Positive control saliva	0.623 ±0.039	0.293 ±0.096

Table 5.3. ELISA results for PA1 saliva assayed with
pre-inoculation saliva (mean ±SD n=8).

		IgA
Pre-inoculation		0.437 ±0.065
Group B		0.533 ±0.153
Group E		0.589 ±0.135
Positive control sera	n=6	0.582 ±0.034
Positive control saliva	n=4	0.504 ±0.030

Table 5.4. ELISA results for PA3 sera samples (mean ±SD n=8).

PA3 IgA

	14 day.	memory.
Group A	0.682 ±0.247	0.487 ±0.096
Group B	0.736 ±0.172	0.534 ±0.077
Group C	0.837 ±0.187	0.576 ±0.023

Pre-inoculation 0.587 ±0.136

Positive control 0.678

PA3 IgG

	14 day.	memory.
Group A	1.120 ±0.488	1.155 ±0.489
Group B	0.794 ±0.216	0.831 ±0.239
Group C	1.129 ±0.403	1.195 ±0.485

Pre-inoculation 0.708 ±0.286

Positive control 1.594

Table 5.5. ELISA results for PA3 saliva samples (mean ±SD n=8).

PA3 IgA

	14 day.	memory.
Group A	0.329 ±0.074	0.593 ±0.088 *
Group B	0.402 ±0.107	0.428 ±0.061
Group C	0.404 ±0.073	0.434 ±0.083

Pre-inoculation 0.411 ±0.067

PA3 IgG

Group A	0.288 ±0.045	0.320 ±0.064
Group B	0.328 ±0.049	0.370 ±0.058
Group C	0.259 ±0.030	0.424 ±0.053

Pre-inoculation 0.332 ±0.071

Table 5.6. Radioimmunoassay results for PA1 sera and saliva samples, counts per minute (mean ±SD n=8).

(i). sera

	IgA	IgG
Group A	1285 ±472	1860 ±601
Group B	1310 ±424	1493 ±509
Group E	1401 ±374	2344 ±633
Positive control	132553	104337

(ii). saliva

	IgA	IgG
Group A	302 ±71	265 ±78
Group B	307 ±116	274 ±89
Group E	378 ±151	340 ±31

Table 5.7. Radioimmunoassay results for PA2 sera and saliva samples, counts per minute (mean +SD n=8).

(i). sera

	IgA	IgG
Group C	838 ±418	1371 ±341
Group D	520 ±108	797 ±186
Positive control	115315	91875

(ii). saliva

	IgA	IgG
Group C	415 ±44	366 ±91
Group D	442 ±157	454 ±91

5.5. Discussion.

The ELISA results from experiments PA1 and PA2 were disappointing in several respects. Firstly, for the immunisation protocols used, the microparticles did not appear to be an effective adjuvant, though, this does not mean that they might not be so when administered according to a different protocol. Secondly, in no group was a significant positive salivary antibody response achieved. However, a small serum response was seen in the control groups, which suggests that small amounts of ovalbumin are absorbed intact after oral administration. Though the site of absorption is unknown. The low levels of circulating antibodies was confirmed in an RIA. A small serum response, following a single oral administration of a soluble antigen has been previously reported in Osborn Mendel rats (28) and following repeated gastric or oral administration in the Wistar rat (151,152). Most workers reporting serum responses in other species have used repeated administration, or long term feeding (149-151).

In considering the salivary response, it is important to note that the blank groups were not true "blanks", since they had received a 20mg dose of ovalbumin by gastric intubation, which was meant as a priming dose only. It is conceivable that this initial

dose raised ovalbumin specific antibodies in the saliva
of all animals in experiments PA1 and PA2 to such a
level that the subsequent repeated intubation of
ovalbumin did not raise the levels further. This could
explain why PA1 animals all showed similiar levels of
salivary antibody. If this is the case, then the
immunisation protocol is poorly designed, the initial
dose is not acting as a priming dose, but as an
effective immunising dose. This possibility was tested,
by re-assay of PA1 saliva, groups B and E alongside
pre-inoculation saliva collected from a separate group
of animals. The results from this assay are shown in
table 5.2. The ELISA values for the groups in PA1
re-assayed, were raised above the pre-inoculation
values, but were not raised significantly. However, the
saliva from PA1 had been repeatedly thawed and
re-frozen since it had been collected, so there is a
distinct possibility that there had been some loss of
IgA binding activity. Therefore, no real conclusions
can be drawn from these results, but the possibility
remains that the oral "priming" dose of 20mg ovalbumin
raised salivary antibodies to such a level, that the
subsequent repeated intubation of 0.5mg per day had no
effect. The non-reliability of the positive controls,
both sera and saliva, for IgA has become apparent.
There is certainly a loss of binding activity of the

IgA antibodies in these samples on repeated assay.

Therefore, in subsequent assays, 35 day sera from

immunisation protocol (ii) section 2.1.3.1. was used as

an IgA positive control.

Effective oral immunisation will result in a

decrease in the subsequent absorption of orally

administered antigen, which could also help to explain

the low salivary responses. In PA2, the booster dose

was administered only two weeks after the repeated

dosing; sufficient time had not elapsed to allow the

production of the cells required to generate a memory

response in the salivary tissue. The booster dose was

also low at only 0.4mg. In retrospect, it was concluded

that the protocol for PA2 was not very effectively

designed.

Another possibility, though a less likely one, is

high dose unresponsiveness in the secretory tissue.

Secretory tolerance to large doses of bacterial

antigens has been reported in mice (148).

The most important reagents in the ELISA are the

second antibodies, the sheep anti-rat immunoglobulins.

The batch here was not identical to that used

previously in assays for salivary antibodies. There is

certainly a possibility of batch to batch variation in

quality, since the reagent is produced by immunising

sheep and is therefore likely to be prone to biological variation in response.

An alternative immunoassay technique was required to either confirm the ELISA data, or to indicate low assay sensitivity. Therefore, an RIA was performed on the samples from PA1 and PA2. The RIA showed good sensitivity and was capable of detecting very low levels of ovalbumin specific antibodies (see chapter 2 for description of method and assay protocol). This work was performed at Beechams Biosciences Research Division, Epsom.

The trends shown with the ELISA for the sera and the saliva responses were confirmed with the RIA. With control groups response > blank and microparticle group responses, and generally blank = microparticle responses. Again, none of the group values were statistically significantly different. This confirms the results shown by the ELISA and demonstrates that this technique is sensitive. Therefore, we were justified in using ELISA further.

Since oral priming failed to produce significant positive salivary immune responses, the rats in experiment PA3 were primed by i.p. injection. Priming by the i.p. route is the best way to achieve an enhanced secretory IgA immune response to subsequent oral challenge with cholera toxin in the rat (155),

although i.p. priming may not be effective for non-bacterial antigens. We were interested in how an i.p. dose might affect the absorption of ovalbumin and how this might affect the serum response.

The older animals used in experiments PA1 and PA2 had not produced good salivary immune responses, so in PA3 younger rats were used. In addition, PA3 was run over a much longer time course, so the larger rats would have become difficult to handle.

The dose administered to the experimental animals by gastric intubation in PA3 was increased. The primary and the memory immune responses could now be assessed in the same group of animals, since they could now be allowed to recover after saliva collection. Pre-inoculation samples could now also be taken, to allow a full assessment of any changes in immune response to OVA.

A booster dose of soluble ovalbumin was administered by gastric intubation, since, although the microparticles may not prove to be an effective adjuvant for a primary immune response, they may alter the memory response in some way.

Prior i.p. challenge was very effective at priming for a positive sera response to orally administered OVA and does not block its absorption across the gut.

Though parenteral immunisation might be expected to decrease the absorption of a subsequently administered antigen. Particulate was as effective as soluble ovalbumin at producing a primary and a memory sera IgG response. Though the sera IgA response to the particulate was less than that of the blank group. These observations raise the possibility of bolstering the serum response to soluble antigens by parenteral immunisation followed by oral administration.

In PA3, particulate antigen produced a significantly better salivary IgA memory response than soluble antigen although the responses after fourteen days were equivalent and did not differ from the blank. Antigen persists for months in the lymphoid tissue of immune animals and is believed to participate in the induction and maintenance of B-cell memory (197). The presence of ovalbumin in an immobilised form, since the particles are not degraded nor translocated to other sites, or the continued leakage of ovalbumin from the particles may be responsible for the enhanced memory response. It is interesting to note that the best salivary IgA response, group A memory response, coincided with the smallest sera IgA response. There is transfer of serum IgA into the salivary glands, though this differs markedly from the transfer into bile in the liver (198). Perhaps the low levels of circulating IgA are a

consequence of the selective transport of serum IgA into mucosal secretions.

These results raise the possibility of producing better secretory immune responses on mucosal re-exposure to antigen, if the antigen was introduced initially as a particulate.

In the next experiment, involving butyl (2-cyanoacrylate) particles, which are biodegradable, the same immunisation protocol as used in PA3 was followed. This work allowed us to assess the effect of particle size on oral adjuvanticity of a particulate dosage form administered orally.

6. POLY (2-BUTYLCYANOACRYLATE) PARTICLES.

6.1. Introduction.

These particles are easily prepared, their size can be altered by physicochemical factors, they adsorb a large variety of materials, are biodegradable and biocompatible. Formation is achieved by aqueous anionic polymerization of butyl 2-cyanoacrylate at low pH in the presence of a steric stabilising agent. The influence of stabilisers on particle size has been investigated (199). Dextran 70 gave particles sized at about 130nm, depending on other physicochemical factors (200), but using beta cyclodextrin it was possible to prepare particles of about 3000nm. The use of these two stabilisers allowed us to assess the effect of particle size on the immune responses to ovalbumin following oral administration adsorbed to a colloidal carrier.

There have been several attempts to improve the bioavailability of drugs after oral administration by association with polyalkylcyanoacrylate particles. Maincent et al (201,202) reported enhanced bioavailability of vincamine after oral administration loaded onto nanoparticles. Initially the transfer of particles across the intestinal barrier was proposed to explain the modified oral absorption, but subsequently the effect was linked to an increase in duration of contact with the gut wall.

Nefzger et al (203) studied the distribution of radioactivity following the oral administration of radiolabelled particles to bile cannulated rats. They reported that 10-15% of the administered radioactivity was absorbed and found in the bile and urine. However, it was suggested that the small amount absorbed probably consisted of low molecular weight components in the polymer and it was concluded that the main part of the nanoparticle material is non-absorbable by rats. Nefzger et al (203) also reported the oral adjuvanticity of nanoparticles for adsorbed inactivated rabies vaccine.

The preparation of nanoparticles directly from insulin and their oral administration has been reported (39). The insulin nanoparticles were biologically active and were claimed to be absorbed intact, although direct evidence of absorption was not provided. Insulin adsorbed onto poly alkylcyanoacrylate particle was not active after oral administration to rats (38). However, passage across the intestine of nanoparticles loaded with insulin after oral administration, with the insulin still biologically active has been reported (204).

The uptake of significant amounts of particle loaded material across the g.i.t. seems unlikely, but uptake of sufficient material to raise an immune response does seem a distinct possibility.

The association of antigens with poly (methyl methacrylate) nanoparticles leads to a good adjuvant effect (205,206) and the particle size of the polymer proved to be an important parameter for adjuvant activity (207,208).

The adsorption of ovalbumin onto butyl 2-cyanoacrylate particles stabilised with dextran 70 and with beta cyclodextrin was assessed, but this involved irreversible aggregation of the particles. Therefore, batches of the particles were prepared by an identical method, mixed with ovalbumin and orally administered to experimental animals.

6.2. Materials and apparatus.

Butyl 2-cyanoacrylate - Henkel, Sichel-werke, Hanover, W.Germany.

Dextran 70 - Sigma.(subsequently termed dextran).

Beta cyclodextrin - Sigma.(subsequently termed cyclodextrin).

Ovalbumin, Grade III - Sigma.

Malvern 2600 laser diffractometer - Malvern Instruments, U.K.

Uvikon 860 spectrophotometer - Kontron.

Sonic bath - Decon FS 100.

MSE High Speed 25.

MSE Centaur 2

Photon correlation spectroscopy.

Autocorrelator - Malvern K7025.

Helium/neon laser - Siemens LGK 7626.

Commodore Pet 2001-32N microprocessor - Commodore, U.S.A.

6.3. Methods.

6.3.1. Particle preparation.

0.25ml of butyl 2-cyanoacrylate was added dropwise to 24.75ml of a filtered (0.2μm membrane, Whatman) aqueous solution of the stabilising agent in

approximately 0.01M HCl at pH 2.25 at 20°C. Dextran

0.5% w/v and cyclodextrin 1% w/v were used separately

as stabilizing agents to prepare different sized

particles.

The preparation was stirred with a glass covered

magnetic stirrer and the rate was adjusted to fully

disperse the monomer. Polymerisation was then allowed

to go to completion over 2 hours. The dextran

stabilised particles were filtered through a sintered

glass funnel (grade 4, pore size 11-16µm).

0.2% w/v poloxamer 188 was added to the particle

suspensions to de-aggregate the cyclodextrin stabilised

particles. Poloxamer 188 was also added to the dextran

stabilised particles to ensure that the two particulate

formulations differed only in particle size, due to the

different stabilisers used in their preparation.

6.3.2. Ovalbumin adsorption.

The particles were fully resuspended by sonication

in a sonic bath, a 10ml aliquot was removed and the pH

was adjusted to 7.4 with the addition of 0.1M NaOH.

Ovalbumin was added and the suspension was stirred for

30 minutes. The suspension was then either intubated

into experimental animals, or the particles were

isolated by centrifugation and the protein adsorption

was assessed by a Lowry assay (see 2.5.3.) on the

supernatant. The protein content of the supernatant was subtracted from the amount of protein added initially to indicate the amount adsorbed to the particles. Cyclodextrin stabilised particles were collected by centrifugation at 4,000rpm for 20 minutes and dextran particles were collected by centrifugation at 21,000rpm for two hours.

6.3.3. Particle size analysis.

6.3.3.1. Photon correlation spectroscopy (PCS).

Dextran stabilised particles were sized by PCS. The instrument used was based on a commercially available Malvern system (fig 6.1.). The sample, held in a thermostatted water bath at $25 \pm 0.05°C$, was illuminated by the laser and the resulting scattered light was detected at $90°$ by a variable angle photon detection system which transmitted the signal to a Malvern K7025, 64-channel, multibit correlator. This was interfaced with a microprocessor to give a print out of the correlation data. Full details of this sizing technique and its validation for the sizing of p(BCA) particles has been described previously from this laboratory (209).

Samples were diluted before measurement with distilled water freshly filtered through a $0.2\mu m$
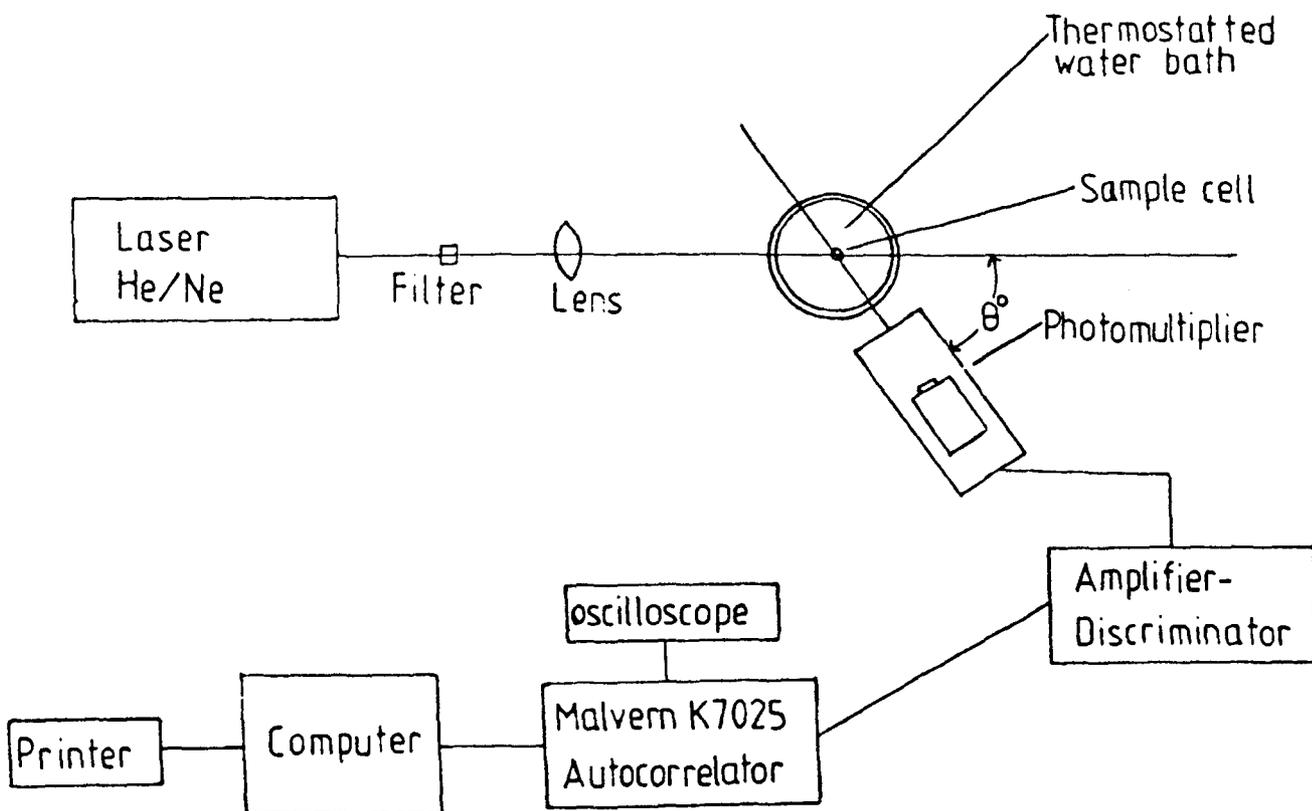
Figure 6.1. Diagramatic representation of photon
correlation spectroscopy apparatus.

membrane filter. The calculated particle diameter and the polydispersity index vary according to the sample time chosen for the size determination, so this was varied to produce optimal conditions. The sample was then analysed 10 times to give an average for the particle diameter and the polydispersity index.

6.3.3.2. Laser diffractometer.

The low power visible laser transmitter produces a parallel monochromatic beam of light which illuminates the particles which are dispersed in the constantly stirred sample cell. The incident light is diffracted by the particles to give a diffraction pattern. The angle of diffraction is dependent on the angle of curvature at the particle surface and therefore dependent on particle size. The diffracted laser light is focused by a Fourier transform lens onto a 31 ring solid state photoelectric detector system. Each ring relates to a particle size and the intensity of light hitting the ring indicates the number of particles of this size. The Fourier transform lens always focuses light from similiar sized particles onto the same ring detector irrespective of the particles position in space. As particles enter and leave the illuminated field the diffraction pattern evolves, always reflecting the instantaneous size distribution in this

area. The ring detector system is interfaced directly
to a desktop computer which reads the diffraction
pattern and performs the necessary integration. The
computer uses the method of non-linear least squares
analysis to find the size distribution which most
closely fits the diffraction pattern obtained (See fig
6.2. for diagramatic representation of this equipment).

6.3.3.3. Coulter counter.

The cyclodextrin stabilised particles were also
sized using a Coulter Counter to confirm the data from
the laser diffractometer. The Coulter Counter was
calibrated and counting performed as described in
chapter 4.

6.3.4. Experimental protocol.

Male Wistar rats of about 200g were used and all the
animals received a priming dose of 5mg OVA in
physiological saline by intraperitoneal injection.
Prior to this, sera and saliva samples were collected
from all the animals and stored frozen. The rats were
then divided into four groups of eight animals which
received the following treatment two weeks later;

Group A received 1mg of ovalbumin in 0.25ml of
cyclodextrin stabilised particles.
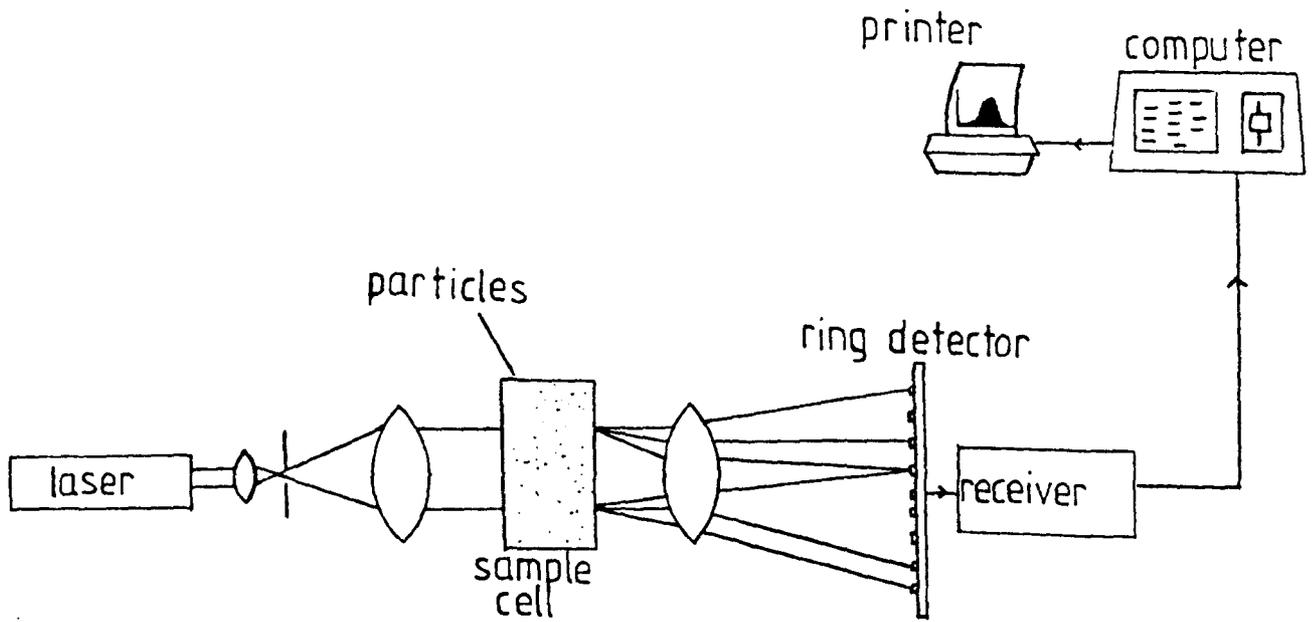
Figure 6.2. Diagramatic representation of laser diffractometry apparatus.

Group B received 0.25ml of a combined suspension of cyclodextrin and dextran stabilised particles.

Group C received 1mg of ovalbumin in 0.25ml of physiological saline.

Group D received 1mg of ovalbumin in 0.25ml of dextran stabilised particles.

It was considered unnecessary to run 'blank' groups for both dextran stabilised and cyclodextrin stabilised particles individually since this would be wasteful of experimental animals and would not provide any more information than could be obtained from having one combined 'blank' group. Therefore, group B was a combined 'blank' group and received g.i. of an equal mixture of both sized particles. All doses were adjusted to pH 7.4 with 0.1M NaOH and were administered by gastric intubation. Each of the intubations for each group of animals was repeated over four consecutive days and sera and saliva samples were collected two weeks after the commencement of the gastric intubations and stored frozen.

Two months after the commencement of the initial intubations, all the animals received a booster dose of 1mg OVA by gastric intubation in physiological saline.

Sera and saliva samples were then collected four days later and stored frozen until assayed by ELISA.

6.4. Results.

6.4.1. Ovalbumin adsorption.

Ovalbumin was efficiently adsorbed to the surface of both cyclodextrin and dextran stabilised p(BCA) particles and adsorption increased as the concentration of the protein was increased (table 6.1.). At a fixed concentration of 4mg/ml, adsorption to the particle surface was reproducible (table 6.2.). The adsorption to cyclodextrin stabilised particles was slightly greater than to dextran stabilised particles (table 6.2.).

6.4.2. Particle size determination.

6.4.2.1. Particle size analysis of dextran stabilised poly 2-butylcyanoacrylate particles by photon correlation spectroscopy.

Ten repeat determinations were performed on the sample, the mean PCS diameter was 215.3nm ±12.0 (mean ±SD n=10), with polydispersity of 0.185 ±0.039. This was converted to an actual particle diameter by a computer program written by Douglas (209), the length number mean diameter was 100.3nm (table 6.3.).

6.4.2.2. Particle size analysis of beta cyclodextrin stabilised poly 2-butylcyanoacrylate particles by laser diffractometry.

Three repeat determinations were performed and the size distributions obtained are shown in table 6.4. The mean sizes obtained were 3.26μm, 3.26μm and 3.25μm.

6.4.2.3. Particle size analysis of beta cyclodextrin stabilised poly 2-butylcyanoacrylate particles by Coulter counting.

Four repeat determinations were performed on the particles and the means of these determinations were plotted (figure 6.3.). The mean particle size was 3.2μm with 25% <2.1μm and 75% <4.25μm.

6.4.3. Enzyme linked immunosorbent assay.

The IgG positive control sample was the same as that used previously in chapter 5, section 5.4.4. for description. The IgA positive control sample was obtained from an animal that was immunised as described in section 2.1.3.1. (ii), the sample was collected after 35 days.

(i). ELISA results for sera samples are shown in table 6.5. Values shown are optical density readings at 405nm for 1/10 dilutions of sera. A significant positive sera

CUMULATIVE
% OVERSIZE.
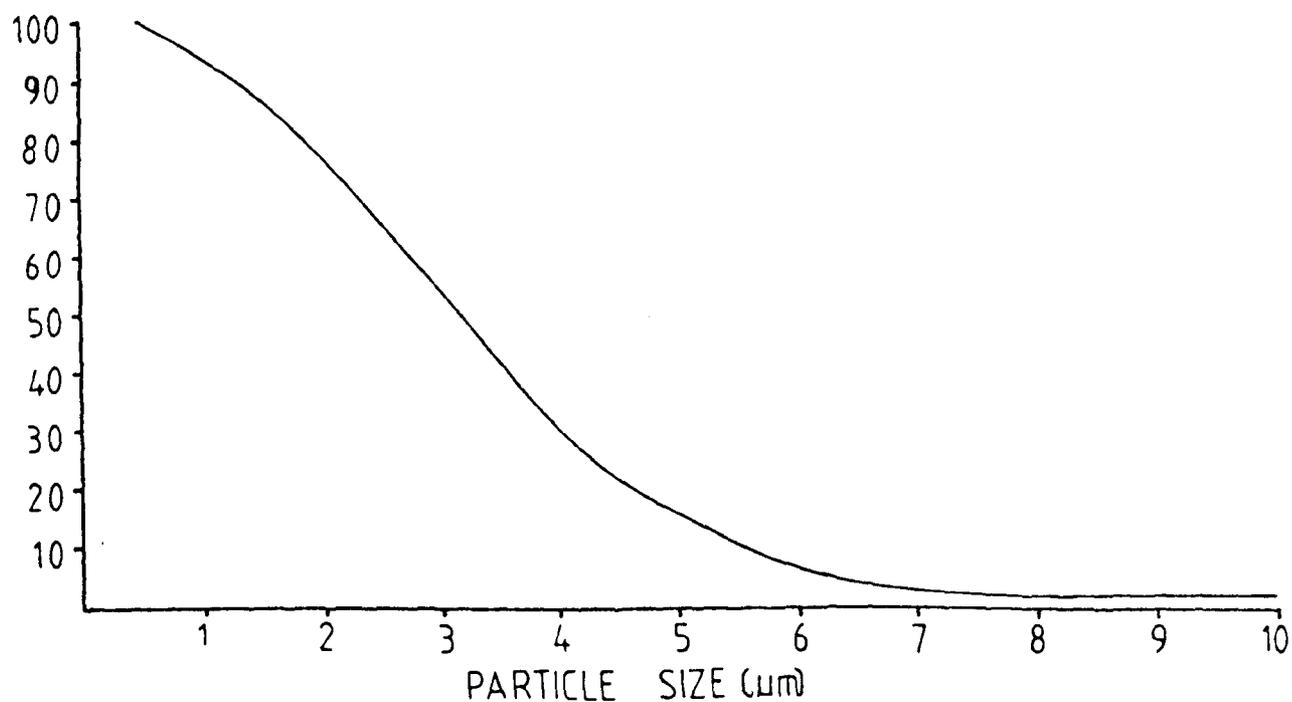
PARTICLE   SIZE (um)

Figure 6.3. Particle size analysis of beta-cyclodextrin
stabilised poly 2-butylcyanoacrylate particles by
Coulter counter.

IgG response was seen after 14 days for group D animals.

(ii). ELISA results for saliva samples are shown in table 6.6. Values shown are optical density readings at 405nm for 1/5 dilutions of saliva. Significant positive salivary IgA responses were seen for both groups A and D after 14 days, and for group D memory response.

Table 6.1. Adsorption of ovalbumin to beta cyclodextrin and dextran 70 stabilised poly 2-butylcyanoacrylate particles.

Ovalbumin added (mg)	beta cyclodextrin stabilised particles Ovalbumin bound		Dextran 70 stabilised particles Ovalbumin bound	
	(mg)	% of added	(mg)	% of added
10	5.36	53.6	6.46	64.6
17.5	10.70	61.4	10.96	62.6
25	16.90	67.6	17.44	69.8
32.5	22.94	70.6	23.68	72.9
40	33.52	83.8	30.10	75.3
47.5	40.90	86.1	36.94	77.8

Table 6.2. Adsorption of ovalbumin at concentration of 4mg/ml to beta cyclodextrin and dextran 70 stabilised poly 2-butylcyanoacrylate particles (mean ±SD n=4).

Ovalbumin added mg.	beta cyclodextrin stabilised particles. Ovalbumin bound		Dextran 70 stabilised particles Ovalbumin bound	
	mg.	% added.	mg.	% added.
40	32.92	82.3	29.32	73.3
40	32.26	80.7	28.96	72.4
40	32.56	81.4	29.14	72.9
40	31.42	78.6	29.02	72.6
Mean	32.29	80.7	29.11	72.8
±SD	±0.640		±0.159	

Table 6.3. Particle size analysis of dextran 70 stabilised poly 2-butylcyanoacrylate particles by photon correlaton spectroscopy (mean n=10).

LN (diameter)	Diameter (nm)	cumulative % undersize
3.0	20.1	.02
3.15	23.3	.08
3.3	27.1	.26
3.45	31.5	.75
3.6	36.6	1.94
3.75	42.5	4.45
3.9	49.4	9.09
4.05	57.4	16.62
4.2	66.7	27.31
4.35	77.5	40.61
4.5	90.0	55.11
4.65	104.6	68.95
4.8	121.5	80.52
4.95	141.2	89.0
5.1	164.0	94.44
5.25	190.6	97.5
5.4	221.4	99.01
5.55	257.2	99.66
5.7	298.9	99.91
5.85	347.2	99.99

Geometric mean diameter = 92.1 nm

Length number mean diameter = 100.3 nm

Table 6.4. Particle size analysis of beta cyclodextrin stabilised poly 2-butylcyanoacrylate particles by laser diffractometry (mean n=3).

cumulative weight % undersize.

Run number

Size (μm)	1.	2.	3.
118.4	100.0	100.0	100.0
54.9	100.0	100.0	100.0
33.7	100.0	100.0	100.0
23.7	100.0	100.0	100.0
17.7	100.0	100.0	100.0
13.6	100.0	100.0	100.0
10.5	100.0	100.0	100.0
8.2	100.0	100.0	100.0
6.4	98.6	98.6	98.6
5.0	88.3	88.3	88.4
3.9	69.3	69.3	69.6
3.0	43.3	43.3	43.3
2.4	21.7	21.7	21.7
1.9	11.7	11.7	11.6
1.5	8.2	8.2	8.1
1.2	6.9	6.9	6.9
Mean size	3.26	3.26	3.25

Table 6.5. ELISA results for BCA sera samples (mean ±SD n=8).

BCA sera IgG.

	14 day response.	Memory response.
Group A	0.730 ±0.385	0.665 ±0.430
Group B	0.485 ±0.063	0.514 ±0.320
Group C	0.416 ±0.114	0.359 ±0.161
Group D	0.681 ±0.050 *	0.426 ± 0.151

Pre-inoculation 0.318 ±0.078

Positive control 2.249 ±0.093

BCA sera IgA.

	14 day response.	Memory response.
Group A	0.542 ±0.278	0.345 ±0.098
Group B	0.369 ±0.191	0.303 ±0.057
Group C	0.285 ±0.038	0.267 ±0.055
Group D	0.336 ±0.017	0.306 ±0.061

Pre-inoculation 0.286 ±0.025

Positive control 1.108 ±0.052

Table 6.6. ELISA results for BCA saliva samples (mean ±SD n=8).

BCA saliva IgG.

	14 day response.	Memory response.
Group A	0.292 ±0.021	0.330 ±0.044
Group B	0.219 ±0.042	0.247 ±0.036
Group C	0.226 ±0.023	0.381 ±0.168
Group D	0.286 ±0.044	0.396 ±0.072

Pre-inoculation 0.291 ±0.045

Positive control 0.937 ±0.121

BCA saliva IgA.

	14 day response.	Memory response.
Group A	0.717 ±0.176 *	0.557 ±0.152
Group B	0.424 ±0.083	0.512 ±0.101
Group C	0.489 ±0.117	0.578 ±0.103
Group D	0.654 ±0.104 *	0.708 ±0.116 *

Pre-inoculation 0.507 ±0.070

Positive control 1.016 ±0.277

6.6. Discussion

The adsorption of OVA to cyclodextrin stabilised particles was slightly greater than to dextran stabilised particles at a concentration of 4mg/ml OVA. This indicates that the stabilising agent, which is exposed at the particle surface (209), has an effect on adsorption. Since otherwise, the smaller dextran stabilised particles, with a much greater surface area available, would be expected to adsorb more material. The efficient adsorption of ovalbumin to p(BCA) particles is a consequence of the high affinity of proteinaceous material for the very hydrophobic particle surface.

It is possible that material adsorbed after oral administration might affect M-cell uptake of the particles in a negative or a positive way, but this is impossible to predict. Immediately after injection into the bloodstream, colloidal particles become coated with various components of the blood (opsonisation). The nature of the surface coating of particles can have a profound effect on their uptake by macrophages and their distribution in vivo. Distribution effects related to particle hydrophobicity/hydrophilicity and the interaction with biological macromolecules may have direct counterparts in the gastrointestinal tract (73).

After oral administration of p(BCA) particles OVA may
be partially displaced on the particle surface due to
competition with other materials. The interaction of
proteins at interfaces is a common phenomenon and when
a non-biological surface contacts whole blood,
immediate adsorption of dissolved plasma proteins and
other substances occurs onto the surface (210). Though,
the overall dose administered to the animals is
unaffected, the possible interactive behaviour of the
particles with dietary constituents, endogenous
bacteria and physiological components of the git,mucins
etc., cannot be predicted. So, it is not known how much
of the ovalbumin remains adsorbed to the particles in
vivo.

The sera IgG response to dextran stabilised
particles was significantly greater than the control
group response after 14 days (significant at p< 0.001
in Unpaired Student "t" test) (table 6.5.). The mean
IgG ELISA value for dextrin stabilised was greater than
that for dextran stabilised particles, but since
the standard deviations were so large, this response
was not significantly enhanced compared to the control
group. This result raises the possibility of inducing
enhanced sera immune responses to orally intubated
antigens, if the antigen is adsorbed to a particulate
carrier. It seems that a more reliable response is

induced if the carrier is sized about 100nm as opposed to about 3μm , though this conclusion is based on a very limited amount of data.

The salivary IgA responses for both dextran and cyclodextrin stabilised particles were significantly greater than the control group response after 14 days (significant at p <0.02 and p <0.01 respectively in Unpaired Student "t" tests when compared with the control group response) (table 6.6). The salivary IgA memory response to dextran stabilised particles was significantly greater than the control group memory response (significant at p<0.05 in Unpaired Student "t" test).

Therefore, as expected, salivary immune responses to OVA were enhanced when OVA was gastrically intubated adsorbed to a particulate carrier. This result is in agreement with the results obtained in chapter 5, although there are important differences in the enhanced salivary response obtained. With p(BCA) particles, significantly enhanced salivary responses were detected after 14 days. This was not so with polyacrylamide microparticles in chapter 5, where only the salivary memory response was enhanced. We feel that this difference in responses to the two particulate carriers is a consequence of the degradation of p(BCA) after uptake into the PP. The high percentage

degradation of p(BCA) and the accompanied release of adsorbed drug over a period of several days has been reported in vitro (211). After uptake, the particle matrix will begin to degrade and adsorbed OVA will be released into the local environment to induce an immune response. As explained in chapter 5, polyacrylamide particles degrade very slowly and OVA is entrapped within the matrix of the particles. Therefore, the particles are likely to remain intact at the site of uptake, and OVA is likely to be released from the microparticles only very slowly. Perhaps then, it is easy to understand why, with polyacrylamide microparticles, the short term salivary immune response is not enhanced, though the memory response is.

Whether the enhanced immune responses seen with p(BCA) particles is a consequence of better uptake of the particles by macrophages etc., slow release of adsorbed OVA into the local environment, or due to enhanced uptake of OVA by the PP since it is adsorbed to particles is unknown. Though it seems likely that a combination of these effects will contribute to the enhanced immune responses seen. The salivary IgA memory response was enhanced for the group which was intubated with dextran   stabilised particles. This probably indicates that at least some of the particle matrix with adsorbed OVA is still intact after a two month

period. Perhaps this result indicates that dextran
stabilised particles are less readily degraded than
cyclodextrin stabilised, though there is no evidence in the
literature to support this.

The results obtained indicate that p(BCA) particles
may be taken up with adsorbed OVA, probably by M-cells,
after gastric intubation. Though we have no direct
evidence of uptake. The enhanced immune responses may
be a consequence of enhanced uptake of OVA associated
with particles, without uptake of the particles
themselves, or the results may be due to protection of
OVA against degradation by the particles, again leading
to enhanced uptake of OVA, but not the particles.
Therefore, an electron microscopy study was undertaken
with gold labelled p(BCA) particles in an attempt to
show directly the uptake of p(BCA) particles by
M-cells.

7. ELECTRON MICROSCOPY.

7.1. Introduction.

To aid in the design of effective peroral vaccines, there is a need for a more fundamental understanding of the various factors governing the uptake of particles by M-cells. One rather limited study on the uptake of several different particulates by PP, indicated that particle surface properties as well as size determines uptake (73). It is therefore of great interest to identify and define the different physicochemical properties of particles which may facilitate their uptake by M-cells. To investigate the physicochemical features that determine uptake in vivo, it is necessary first to be able to identify M-cells and secondly to quantify the uptake process. This chapter reports initial work to identify M-cells in the follicle associated epithelium overlying a PP in the rat small intestine. To aid this identification, p(BCA) particles were labelled with colloidal gold and introduced into a gut loop prepared around a grossly visible PP in the rat ileum. The uptake of p(BCA) particles by the M-cells would provide evidence that these particles are suitable candidates for oral immunisation studies.

In order to study the tissue in question under the electron microscope, a detailed process of fixation and

processing is required to prepare the tissue. The neccesary general steps required in the process of electron histology are as follows:

A. Fixation: killing the tissue, but at the same time preserving faithfully all the tissue fine structure as near as possible to its form in life.

B. Block staining: rendering the preserved fine structure visible in the electron microscope by causing certain components to attract heavy metal ions and thus to scatter electrons differentially.

C. Dehydration: remove water from tissue and replace it with inert fluid miscible with water and infiltrating fluid.

D. Infiltration: infiltrating fluid introduced which can be easily hardened into an elastic solid.

E. Polymerisation: hardening of infiltration fluid to produce a solid matrix to support tissue.

F. Sectionning: slicing the block into ultra-thin sections <600Å thick.

G. Mounting: transferring different sections to copper support grids for insertion into the electron microscope.

H. Section staining: increasing the existing differential electron scattering power (contrast) of the tissue constituents by reacting the mounted sections with heavy metal solutions.

The tissue sections are now ready for examination in
the electron microscope.

7.2. Materials and apparatus.

Pentobarbitone sodium 60mg/ml - 'Sagatal' M+B
veterinary, Dagenham, Kent.

Primary fixative - 2% glutaraldehyde in 0.1M phosphate
buffer pH 7.2.

Glutaraldehyde EM 25% - TAAB Laboratories Ltd, Reading,
Berks.

Osmium tetroxide in Millonigs buffer

   solution A. 2.26g $NaH_2PO_4 . 2H_2O$ in 100ml water.

   solution B. 2.52% NaOH.

   solution C. 5.4% glucose in water.

   Approximately 15ml of solution B was added to 75ml
of solution A until pH 7.2-7.4 was reached and 1g of
Osmium tetroxide was dissolved in this, 10ml of
solution C was then added to produce the fixative.

Osmium tetroxide - JMC, Royston, Herts.

Propylene oxide - Fisons, Loughborough.

Embedding medium

   Equal parts of Araldite monomer and hardener DDSA
with 1 drop of accelerator BDMA for each ml of
embedding medium used. The plastics were thoroughly
mixed before use.

Epoxy resin Araldite CY212 - TAAB.

DDSA dodecyl succinic anhydride hardener 964, EM grade
   - TAAB.

BDMA benzyldimethylamine - TAAB.

Toluidine blue stain - 1% toluidine blue in 0.05M

phosphate buffer pH 7.2.

Lead citrate (Reynolds) stain kit - EM scope Labs.

Ltd, Ashford, Kent.

Automatic micro-osmometer - Roebling, Berlin, W.G.

LKB 7800 Knifemaker.

Huxley ultramicrotome - Cambridge.

Electron microscope EM 410 Phillips

7.3. Methods.

7.3.1. Preparation of colloidal gold containing poly

butyl cyanoacrylate particles.(This work was performed

on my behalf by my colleague J.Wright.)

Colloidal gold was prepared according to the method

of Frens (212), 1ml chloroauric acid in 50ml double

distilled water was heated to boiling 2ml trisodium

citrate (1%) was added and the solution boiled for 5

minutes. The solution rapidly turned blue and then red

to indicate that particle formation was complete, a

size analysis was then performed by PCS.

Four ml of colloidal gold was incubated for 15

minutes with 40mg Pluronic F127, to stabilise the

colloid, and was then added to 20.75ml of dextran 70 1%

w/v in 0.01M HCl pH 2.25. Poly 2-butyl cyanoacrylate

particles were prepared according to the method of Douglas et al (200), outlined in section 6.3.1.

7.3.2. Gut loop preparation.

One male Wistar rat of about 200g was used, which was fasted overnight. The animal was anaesthetized by i.p. injection of Sagatal 60mg/Kg body weight and the abdominal cavity was entered. A PP was identified on the serosal side of the intestine and a 5cm gut loop was prepared around this structure.0.5ml of the particle suspension was injected into this gut loop and the intestines were replaced in the abdominal cavity which was resealed with a clamp.

About 30 minutes later, the gut loop was removed from the abdominal cavity and immersed in the primary fixative. Under a dissecting microscope, the PP was removed from the surrounding mucosal tissue with the aid of a needle and placed in fresh primary fixative for 3 hours.

7.3.3. Tissue processing.

The tissue was placed in the primary fixative for 3 hours at room temperature, which was then pipetted off and replaced with 0.1M phosphate buffer pH 7.2 containing 4% sucrose. The buffer solution was then

replaced with further buffer and the tissue was allowed to soak at room temperature overnight. The tissue was then further washed by replacement of the buffer, which was in turn replaced by Osmium tetroxide solution for tissue staining. The tissue remained in Osmium tetroxide solution for 1 hour, then rinsed in 50% ethanol. Dehydration of the tissue was achieved with successive replacement of the 50% ethanol with 70%, 90% and then 100% ethanol. At each stage, the tissue was allowed to equilibrate in the ethanol for about 15 minutes. The tissue was then placed in propylene oxide for two 15 minute periods before being left overnight in a 50/50 solution of propylene oxide and embedding medium at room temperature. This solution was replaced with full strength embedding medium and left for 24 hours at room temperature, before being placed in rounded moulds and polymerised for 7 days at 60°C.

Thick plastic sections stained with toluidine blue were prepared and viewed under the light microscope to establish the orientation of the tissue. Ultrathin sections were then cut from the block, mounted on copper grids and stained with lead citrate prior to examination in the EM 400.

General reference (213).

-193-

7.4. Results.

7.4.1. Particle size analysis.

(i). Colloidal gold.

Nine repeat determinations were performed on the colloidal gold sample to be incorporated into the p(BCA) particles. The mean PCS diameter was 35.87 ±6.21nm (mean ±SD n=9), with polydispersity of 0.376 ±0.087. This was converted to an actual particle diameter as described in section 6.4.2.1., the length number mean diameter was 8.8nm (table 7.1.).

(ii). Poly 2-butylcyanoacrylate particles.

Nine repeat determinations were performed on the particles, the mean PCS diameter was 208.4 ±6.28nm (mean ±SD n=9), with polydispersity of 0.101 ±0.048. This was converted to an actual particle diameter as described in section 6.4.2.1., the length number mean diameter was 135.1nm (table 7.2.).

7.4.2. Electron micrographs of putative M-cells.

Electron micrographs of two putative M-cells are shown in figures 7.1. and 7.2. at magnification of x6,485 and x11,923 respectively.

Table 7.1. Particle size analysis of colloidal gold by

photon correlation spectroscopy (mean n=9).

LN (diameter)	Diameter (nm)	cumulative % undersize
1.5	4.5	8.16
1.65	5.2	18.25
1.8	6.0	29.85
1.95	7.0	42.27
2.1	8.2	54.64
2.25	9.5	66.10
2.4	11.0	75.99
2.55	12.8	83.93
2.7	14.9	93.98
2.85	17.3	96.64
3.0	20.1	98.24
3.15	23.3	99.14
3.3	27.1	99.84
3.45	31.5	99.94
3.6	36.6	99.98
3.75	42.5	100.00

Geometric mean diameter = 7.5 nm

Length number mean diameter = 8.8 nm

Table 7.2. Particle size analysis of butyl

2-cyanoacrylate particles, with incorporated colloidal

gold, by photon correlation spectroscopy (mean n=9).

LN (diameter)	Diameter (nm)	cumulative % undersize
3.6	36.6	0.01
3.75	42.5	0.05
3.9	49.4	0.24
4.05	57.4	1.00
4.2	66.7	3.37
4.35	77.5	9.26
4.5	90.0	20.83
4.65	104.6	38.82
4.8	121.5	60.97
4.95	141.5	82.55
5.1	164.0	99.19
5.25	190.6	100.00

Geometric mean diameter = 128.8 nm

Length number mean diameter = 135.1 nm

Figure 7.1. Electron micrograph of putative M-cell in
the epithelium overlying the Peyer's patch in a Wistar
rat.   x 6,485.
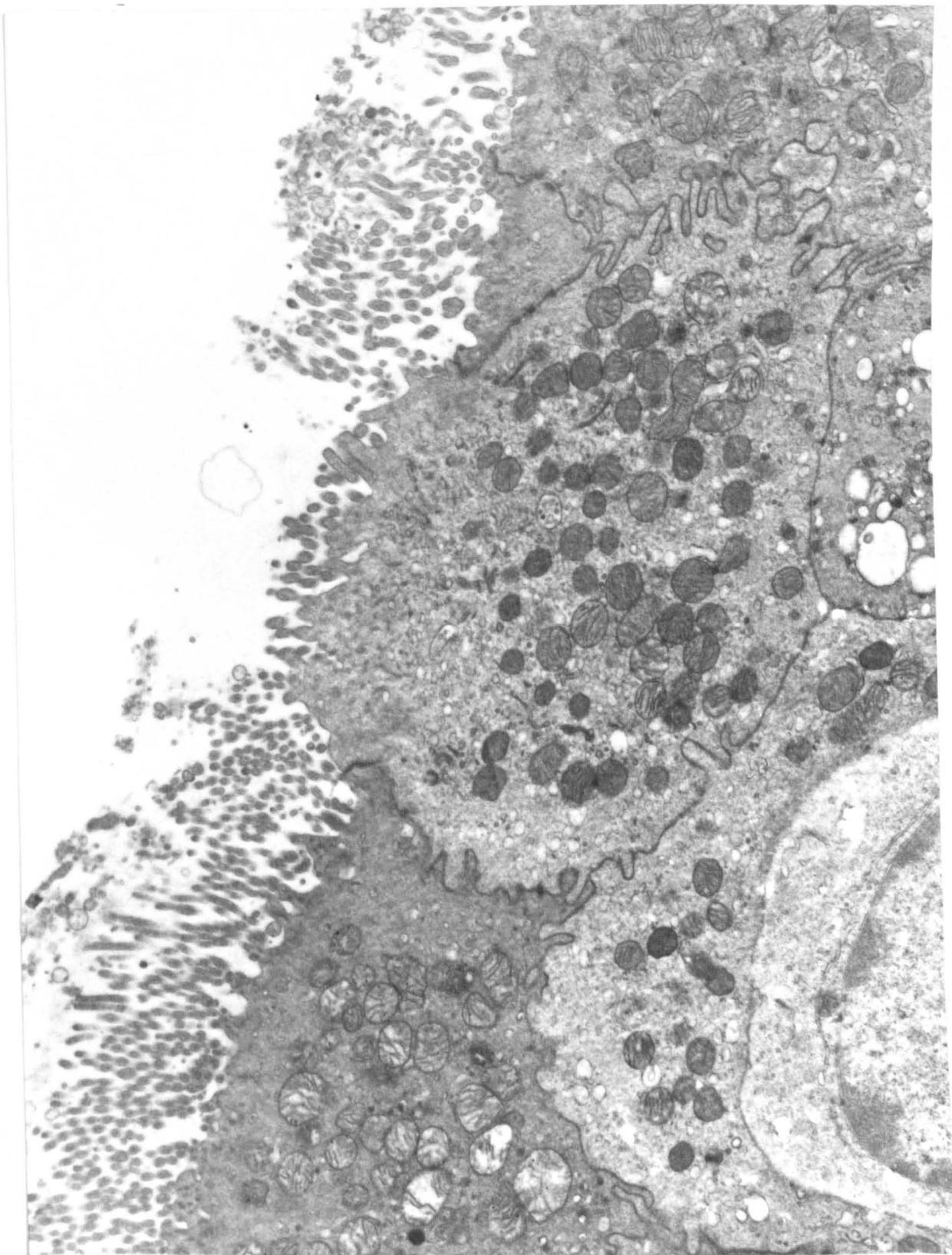
Figure 7.2. Electron micrograph of putative M-cell in
the epithelium overlying the Peyer's patch in a Wistar
rat.   x 11,923.

7.5. Discussion.

The cells could not be identified under light microscopy and a careful study of the PP tissue in the electron microscope was required. But this was mainly due to the very limited amount of epithelial tissue available to study in the section. The orientation of the PP in the block could only be controlled with great difficulty since the specimen was so small, about 2mm. So it is left to chance to govern what angle the PP was sectioned. Consequently, the specimen may not necessarily have been sectioned at an angle appropriate to the inclusion of much epithelial tissue in the section. It was very easy to lose some tissue from the specimen during removal, processing etc. and any lost tissue was likely to be the epithelial tissue since this tissue is surface located and is easily shed. Therefore, it is easy to see why there was so little epithelial tissue present in the section.

Since the orientation of the tissue in the block could not be controlled, this led to sectioning at an oblique angle which further complicated the identification of M-cells. However, when a section of epithelium that had not been cut at a particularly oblique angle was identified under the microscope, the subsequent identification of the putative M-cells was

not very difficult. The epithelium was examined systematically until a cell appeared whose microvilli were noticeably different from those of the adjacent ordinary epithelial cells. The cells were then photographed for closer study.

Putative M-cells were identified in the epithelium overlying the PP, figs 7.1 and 7.2. These cells displayed a number of characteristic features which have been ascribed to M-cells by several workers, they did not display regular 'finger-like' microvilli on their apical surface, like ordinary absorptive cells, but displayed shorter irregular microvilli (85-87). The cells had a thin bridge of apical cytoplasm, with abundant vesicles, fig 7.2, and the cytoplasm surrounded several intrusive cells which appeared to be lymphocytes (87). These intrusive cells are not within the M-cell cytoplasm, but in the intercellular space that indents the M-cell cytoplasm, forming a "central hollow" (80). The cells displayed less electron dense cytoplasm and more electron dense mitochondria than the adjacent ordinary epithelial cells (214). Therefore, although it cannot be stated categorically that these cells identified in the epithelium overlying the PP are M-cells, all the available evidence would suggest that they are.

No particles were observed associated with the
epithelial surface and none were observed in M-cells.
This may indicate that insufficient colloidal gold was
incorporated into the particles to render them visible
in the microscope, or it may indicate that there is a
fundamental flaw in the experimental technique. It
seems more likely that the gold incorporation technique
was unsuccessful.

8. Final discussion and conclusions.

The overall aim of this project was to enhance immune responses to the chosen model protein following oral administration. To achieve this the model protein was associated with colloidal carriers. We were particularly interested in the salivary IgA response, however, a number of problems were encountered in the determination of salivary IgA by ELISA. Rat IgA could not be obtained commercially and as a result it was impossible to perform a quantitative assessment of the ELISA technique. Also, unfortunately, the quality of the sheep anti rat IgA conjugate, the most important reagent in the ELISA, could not be determined. The only option open to us, was to initially prove the assay system capable of detecting IgA in the serum, and then move on to assay salivary samples using the same protocol. However, there are drawbacks to this approach. A serum IgA response cannot be induced without stimulating the production of other immunoglobulin isotypes, particulary IgG, which is much more abundant in the serum than IgA. Therefore, in the serum, there are many anti-OVA antibodies, but only a small proportion of these are IgA. So in the ELISA, the anti-IgA conjugate has relatively few sites with which to combine specifically. Lack of specificity of this

reagent will result in binding to other more abundant isotypes and will lead to false positive results. Therefore, the research was very dependent on the quality of one reagent, which is produced biologically, and whose quality cannot be assessed. However, the supplier's literature states that the conjugates are "specific for the respective rat immunoglobulin proteins when tested by immunodiffusion and immunoelectrophoresis against a variety of rat sera and other immunoglobulins". Although, of course, it would be more satisfactory if the reagents were affinity purified, but none was available.

Serum IgA may not necessarily have identical binding properties to salivary IgA, since secretory component and other factors in secretions may interfere with antigen-antibody interactions. However, since IgA is not available commercially, no assessment can be made of the possible inhibitory effects of salivary components. Whilst recognising the drawbacks of this approach, the ELISA was validated by a demonstration of its ability to determine rat antibodies in serum, both IgA and IgG, following i.p. injection of the model protein. The detection of antibodies in the saliva proved to be a problem. Several immunisation protocols proved ineffective at inducing a salivary response. A response to myoglobin was eventually induced only when

it was administered orally on four consecutive days, followed by a 'booster' oral dose, more than two months later. Ovalbumin, administered at a higher dose, induced a significant salivary response after oral administration over four consecutive days only. However, subsequent data did bring into question the interpretation of these results as 'significantly positive' as the response was compared with the salivary response from only two "blank" animals. This limitation in experimental design is explained by our reluctance to use a large number of experimental animals at this early stage. We were unsure about the effectiveness of this immunisation protocol, but needed to demonstrate that at least a small salivary response would be induced, so that we could then move onto the next stage of the work and try and enhance this. The response induced seemed to justify moving onto the next stage, the oral intubation of OVA associated with the chosen colloidal carriers.

The simple and accurate technique of Lowry proved applicable to the quantitative determination of proteins incorporated into or adsorbed onto liposomes and p(BCA) particles respectively. The adsorbed protein could not be recovered from p(BCA) particles, so the amount left in the supernatant after collection of the particles was assayed and the value subtracted from

that added initially. The entrapment of protein in polyacrylamide microparticles was determined by HPLC after hydrolysis and derivatization of the constituent amino acids.

Oral administration of ovalbumin incorporated into DSPC/CHOL liposomes led to slightly enhanced immune responses, both in the serum and the saliva. But the enhancement was not statistically significant. However, since both IgA and IgG responses were enhanced in both serum and saliva, it seems less likely that these values could have arisen purely by chance. The results raise the possibility that, perhaps with a different immunisation protocol, or for a different antigen, DSPC/CHOL liposomes may prove an effective oral adjuvant. However, the recently reported lability of DSPC/CHOL liposomes in simulated intestinal media and the reported lack of absorption of entrapped markers by intestinal cells (166) may belie this note of optimism.

The volume of saliva collected from the liposomal experimental animals was recorded, to ascertain if this would greatly affect the ELISA result. The volume collected from each animal varied from 300-500μl (mean 388 ±55μl), but since the ELISA values varied so little, these values were of no consequence. It was concluded that the immunisation protocol used was innappropriate to the induction of a significantly

positive salivary immune response. Which indicated that we had not elicited a significant positive salivary antibody response when this immunisation protocol had been used previously in chapter 3. As explained, this response had been compared with only two 'blank' samples at the time. This work could have been repeated, with more 'blank' samples, but instead the immunisation protocol was expanded for subsequent experiments to also include an oral priming dose of OVA. Therefore, in the subsequent experiment, involving polyacrylamide microparticles, which appeared to have greater potential as an oral adjuvant, an oral priming dose of ovalbumin was administered.

Initially, two experiments with polyacrylamide microparticles were performed, PA1 and PA2; with PA2 being a continuation of PA1 designed to show the effect of a booster dose of antigen administered orally, in particulate and soluble form, two weeks after the commencement of the repeated oral administration. Unfortunately, the results from PA1 and PA2 were very disappointing, no significantly positive responses were seen in the serum or the saliva of any group of animals. A radioimmunoassay, proven to be capable of detecting both IgG and IgA rat anti-OVA antibodies, indicated that the negative results were not due to lack of sensitivity of the ELISA. Therefore, it was

concluded that the immunisation protocols for PA1 and PA2 were poorly designed, despite being based on literature reports and our own previous results.

In experiment PA3, an intraperitoneal priming dose was administered to the animals and, since the necessary licence had been obtained from the Home Office, sequential sampling was performed, to allow us to assess fully any changes in the immune responses. The time course of this experiment was extended so that the 'memory' response to oral administration of antigen more than two months after the initial intubation could be assessed. In previous work, the memory response was considerably larger than the primary response. Six capillary tubes of saliva was collected from each animal at each collection point, which constituted a total of over 200μl.

After 14 days, the group mean salivary responses did not differ and were not raised above the pre-inoculation levels. However, the mean serum IgG response for both the particulate and the control group was raised, as was the control group serum IgA response. But the large degree of variation within the groups meant that the control and the particulate group responses were not raised significantly above the blank group value. This degree of variation within groups is

common when biological responses are assessed from a number of unrelated individuals.

The mean memory salivary IgA response of the particulate was significantly raised ($p < 0.01$ in Unpaired Student "t" test) relative to the control group and there was much less inherent variation in the group values than was seen for the serum response. This result raises the possibility of inducing enhanced and more reliable salivary immune responses on re-exposure to antigen, if the antigen is administered initially as a particulate. The persistent presence of the antigen in the tissues, in an immobilised form, after uptake via the PP was postulated as an explanation for the enhanced salivary response.

Both control and microparticulate group mean serum IgG responses remained higher than the blank for the memory response, but again, there was large variation within the groups.

It can be concluded from these data that the oral administration of ovalbumin incorporated in polyacrylamide microparticles can lead to an enhanced secretory immune response, depending on the immunisation protocol used. Other microparticles, which either confer protection against the degradative properties of the gut on an entrapped antigen, and/or promote uptake by PP, might be expected to display a

similiar adjuvanticity. More biodegradable and biocompatible systems seem appropriate for further studies, although, the non-biodegradability of polyacrylamide microparticles may be of paramount importance to the oral adjuvanticity.

The immunisation protocol used in PA3, was repeated for a further experiment involving p(BCA) particles with adsorbed ovalbumin. Two groups of animals were given particles, which differed only in particle size, due to the different stabilising agents used in their preparation. It was demonstrated that ovalbumin was efficiently adsorbed to the particles (70-80% at 4mg/ml ovalbumin) in vitro, and that the adsorption was reproducible. However, it cannot be predicted how much ovalbumin would remain adsorbed to the particles in vivo, after oral administration. There is likely to be competitive displacement of ovalbumin at the particle surface due$_\wedge$the adsorption of proteins and other materials present in the gut, analogous to the adsorption of blood components onto non-biological interfaces when combined (210).

The 14 day sera IgG response in the group administered OVA adsorbed to poly 2-butylcyanoacrylate particles stabilised by   dextran   was significantly raised relative to the control group response (significant at p <0.001 in Unpaired Student

"t" test). Although the mean ELISA value for the group administeredcyclodextrinstabilised particles was greater than the value obtained for the aforementioned particle group, the large amount of variation in the response meant that the sera response was not significantly raised above the control group response. These results raise the possibility of inducing better sera immune responses to an orally administered antigen, if the antigen is adsorbed to a particulate carrier. It seems a more reliable response is obtained if the carrier has a size of about100nm as opposed to :3μm .

The 14 day saliva IgA response was raised significantly relative to the control groups for both groups administered OVA adsorbed to particles, beta cyclodextrin and dextran 70 stabilised (significant at p <0.01 and p <0.02 respectively in Unpaired Student "t" test). The saliva IgA memory response for the group administered   dextran stabilised particles was significantly raised above the control group response (significant at p <0.05 in Unpaired Student "t" test). These results raise the possibility of inducing better secretory immune responses to orally administered antigens if the antigens are adsorbed to particulate carriers. Better memory responses in the secretory tissue may also be elicited with a particulate carrier.

It was thought that the enhanced immune responses after 14 days are a consequence of both the degradation of the particles after uptake into the PP, which will cause release of adsorbed OVA, and also desorption of OVA from intact particle. Both of these will result in the release of OVA over a quite short time period and so are likely to result in an enhanced short term immune response. The better saliva IgA memory response from the group administered   dextran stabilised particles is likely to be a consequence of particle size. The small particle size, compared to cyclodextrin stabilised particles, or possibly the dextran   component, may make the particles more difficult to degrade in vivo eg. uptake by macrophages etc. may be less efficient.

The results from chapters 5 and 6 indicate that particulate carriers can gain access to the PP tissue after oral administration and can result in enhanced immune responses to an incorporated, or adsorbed antigen. But there was no direct evidence to show the uptake of these carriers after oral administration. The next part of this work was undertaken to rectify this.

The electron microscopy study was undertaken ostensibly to indicate that p(BCA) particles are a suitable candidate for oral immunisation studies by virtue of their in situ uptake into M-cells. But the

gold labelled particles were not sufficiently electron dense to allow their visualization in the tissue sections. However, it was possible to identify putative M-cells in the epithelium overlying the PP, despite the colloidal particles themselves not being visible.

The fundamental problem encountered in this research work, was the unreliable and non-reproduceable nature of the salivary immune response. In part, this may be explained by interference of antigen-antibody reactions in the ELISA by salivary components, by loss of antibody activity on freezing and thawing, by batch to batch variation in the quality of the anti-rat IgA conjugate, or perhaps low reactivity of this reagent with salivary IgA. However, although the 'common mucosal immune system' was conceived to include salivary and mammary glands (137), subsequent evidence showed that intestinal lymphoblasts are primarily tissue-specific and hence migrate to these other mucosal sites in much smaller numbers (214). This perhaps offers a better explanation of the poor salivary immune response to gastrically administered antigens, but does not invalidate the measurement of the secretory immune response from the saliva. However, a direct determination of the intestinal antibody response would be preferred if possible.

Overall, we feel that this work offers encouragement towards the further investigation of the potential of microparticulate systems as orally administered immunological adjuvants.

9. Future work.

Standard methods for protecting antigens against degradation in the gut eg. enteric coating, or concomitant administration of sodium bicarbonate, will not be discussed here, since these methods are well established and have been employed in large scale clinical trials in man for oral immunisation against typhoid fever (215,216).

As an aid to the design of peroral vaccines based on polymeric particles, a more fundamental understanding of the factors governing the uptake and transport of particulates by the PP would be of great benefit. It is felt that a detailed study should be undertaken, with a wide range of particles of known size, surface charge, hydrophobicity etc. to determine exactly which surface properties render the particles more attractive for uptake by M-cells. However, although characterisation of the surface properties governing uptake would be of great benefit, the effects of adsorbed or incorporated antigen and the effects of material adsorbed to the particles in the gut must also be considered. To allow their visualization in the electron microscope, the particles must first be labelled to render them sufficiently electron dense, ferritin offers promise as

an effective label which has been shown to be taken up by PP.

The quantification of the uptake of particles into the PP may prove difficult, but labelling particles with radioisotopes and subsequent counting of activity in excised PP tissue could solve this problem. The fate of particles in subjacent tissues could also be followed using microautoradiographic techniques. The processing of particulates by dendritic cells, macrophages and lymphocytes may be dependent on characteristics such as size, surface charge, hydrophobicity etc. The induction of a positive secretory immune response will depend on the effective processing of antigens by these cells and microautoradiographic studies may help to clarify this complex intracellular and intercellular processing.

Much may be learnt about the uptake and transport of materials by M-cells by the study of the uptake and transport of bacteria and viruses. The bacterium Vibrio cholerae is taken up and transported by M-cells (97,99) and the process must be active, since the organism is non-invasive. Chlamydia have been observed in the M-cells of calves with diarrhoea, but transport in PP has yet to be demonstrated (217). More interestingly, certain organisms, such as RDEC-1 Escherichia coli and Reovirus type 1, seem to bind preferentially to M-cells

(95,99,105). RDEC-1 attaches to M-cells long before it manifests general colonization of the mucosal surface (105). Adherence was found to be dependent on a plasmid encoded pilus, which suggests that M-cell binding is at least part dependent on the presence of certain M-cell specific surface molecules (218). Reovirus binding to absorptive cells is dependent on a surface protein different to the one responsible for binding to M-cells (96). These observations raise the possibility of M-cell binding factors, which, if successfully identified and isolated may provide a specific mechanism for targetting to M-cells. The RDEC-1 E.coli strain, may be grown in media to promote pilus production, the pilus may then be isolated and linked directly to an antigen, or possibly linked onto the surface of a colloidal carrier incorporating the antigen.

Differences in M-cell apical membrane may also offer possibilities for targeting (107-9). The ability of M-cells to act as an entry route for the uptake of materials into the PP, will depend not only on the binding characteristics of the cell, but also on the ability to endocytose material. Two strains of E.coli which have the same ability to bind to M-cells, differ in their capacity to be transported into the lymphoid follicle (219).

Concerning the non-reliability and non-reproduceability of the salivary IgA response, in future experiments, the small intestine, from the duodenum to the ileocaecal junction, could be removed, homogenised and the intestinal antibody isolated. This method would allow a direct determination of the gut immune response, which would be of particular importance when immunising against intestinal pathogens. Furthermore we could still assess the on going immune response by sequential salivary collection. An assessment of the cellular immune response could be performed by a lymphocyte proliferation assay in the presence of the immunising antigen, after collection of mesenteric lymph and isolation of the lymphocytes.

We considered that future work should be directly concerned with the causative agents of enteric, and possibly other mucosal-site, infections and not with model antigens. It should be remembered that, since lymphocytes seed out to other mucosal sites after oral administration, peroral vaccination may also elicit protection against respiratory pathogens such as Haemophilus influenzae and Bordetella pertussis or uro-genital pathogens such as Neisseria gonorrhea.

Studies with cholera have revealed the important principle of synergism between antigens interfering

with different pathogenic events such as colonization
and toxin action (220). This principle has been amply
supported for the action of anti-CFA (colonization
factor antigens) and anti-LT (heat labile enterotoxin)
antibodies in protection against experimental
enterotoxigenic E.coli infections (221). Therefore,
future work in the development of enteric vaccines
should be aimed towards the establishment of
synergistic cooperation.

Again, it seems that much can be learnt by the study
of the mode of action of microbiological agents.
Cholera toxin is an excellent mucosal immunogen,
probably both due to its ability to bind to cell
membrane GM1 ganglioside receptors, which may be
present on M-cells and its ability to activate
adenylate cyclase in lymphoid tissue. Cholera toxin has
the ability to markedly enhance the response to
unrelated antigens (222). In fact, two synthetic
peptides of only 20 and 25 amino acids respectively,
corresponding to peptide sequences in the cholera toxin
B chain, have been shown to be effective adjuvants when
given orally or intraperitoneally (223). These
particular sequences were chosen because of their
predicted surface localization. Perhaps the most
efficient way of inducing a mucosal immune response
will prove to be through linking of synthetic peptide

sequences, like those from cholera toxin, with the proposed antigen, incorporated into a protective colloidal carrier. Cholera toxin is proposed to work in vivo by providing extra help in the induction of precursor B cells in PP by mimicking or replacing the action of T helper cells and at the same time inhibiting the induction and /or function of T suppressor cells. An appropriate colloidal carrier would be chosen to ensure that the cholera toxin gains effective access to the PP.

There are several, perhaps less feasible approaches to enhancing immune resonses to orally administered antigens. Firstly, linking antigens to soluble, cross linked N-(2-hydroxypropyl) methacrylamide copolymers. These polymers have been shown to be pinocytosed by rat intestine in vitro, with the rate of uptake increasing with increasing size of the polymer (224). Secondly, encouraging the lymphatic absorption of antigens eg. by their attachment to high molecular weight dextran carriers (225). Certain large molecular weight toxins, such as botulism toxin, have been found in significant levels in the lymph (47). Thirdly, a greater understanding of receptor mediated and facilitated transport mechanisms dealing with dietary materials eg. receptor mediated endocytosis of vitamin B12 (53-56), may offer possibilities for the enhanced uptake of

suitably tailored molecules. Fourthly, another possibility may be to produce particles directly from antigens. Insulin nanoparticles were reportedly active after oral administration (39) and particles may be prepared by the coacervation of many macromolecules (226). Although all of these approaches have serious limitations, in some situations they may prove applicable and are worthy of consideration.

This thesis has dealt only with the potential of pharmaceutical formulations as oral adjuvants, there is undoubtably great scope for further research into the potential of pharmaceutical formulations as adjuvants for other modes of administration. A recent review paper by Allison (227) shows the rational development of a very promising injectable adjuvant formulation, developed following sound pharmaceutical reasoning. Perhaps this will prove to be the first of many such developed vaccines. There is no indication that this adjuvant is effective orally.

It has been suggested that the human immunodeficiency virus (HIV) gains access to the body via the M-cells in the lymphoid follicle rich rectum (228). If this is so, then possibly oral immunisation with a killed, or inactivated portion of the virus in an effective adjuvant may provide the first line of defence against this highly dangerous pathogen.

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