AN INVESTIGATION INTO THE IMMUNE RESPONSE PRODUCED BY DIETARY PROTEINS

By

JOCELYN ROSEMARY NOTLEY, B.Sc.

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ABSTRACT

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In order to understand the mechanisms involved in adverse reactions to food proteins which are mediated by the immune system it is necessary to understand the normal immune response following the ingestion of dietary proteins. Thus, the effect of age, source and quantity of protein species of animal and maternal influences on this response have been examined.

Introduction of a novel dietary protein to the diet of mature rabbits resulted in a greater immune response compared to the weanling, indicating absorbtion of antigenic protein in sufficient quantities to stimulate the production of circulating antibodies is not only a function of the immature intestine.

Production of serum specific antibodies is influenced by the duration of antigenic exposure, source and quantity of ingested protein. The magnitude of the immune response appeared to be due to the quantity of antigenic protein reaching the circulation.

Specific systemic hyporesponsiveness was observed in rabbits following maternal exposure to the dietary protein. This was also influenced by the quantity of ingested protein and duration of antigenic exposure. In contrast, this treatment did not produce systemic tolerance in the mouse, instead the offspring demonstrated high levels of specific serum antibodies.

The mouse differs from the rabbit and human by receiving the majority of passive immunity post-natally. Thus, the systemic immune response observed in the weanling mouse is influenced by maternal antibodies.

Acute ingestion of cows milk and goats milk by human volunteers did not result in increased levels of specific circulating antigen or Clq binding immune complexes in the majority of volunteers. This suggests that the intestine provides an efficient barrier against the absorbtion of large quantities of antigenic protein in most adults.

A certain amount of evidence suggests that immune mechanisms play a role in the pathogenesis of atherosclerosis; however, the immune responses in rabbits following the ingestion of a milk based diet did not influence the development of atherosclerosis.

TO MY PARENTS

WITH LOVE

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ABBREVIATIONS

B-Cell	Bone marrow derived lymphocyte
T-Cell	Thymus derived lymphocyte
lgG	Immunoglobulin G
lgA	Immunoglobulin A
lgM	Immunoglobulin M
DTH	Delayed-type hypersensitivity
PFC	Plaque forming cell
GALT	Gut associated lymphoid tissue
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme-linked-immunosorbant assay
AHG	Aggregated human gamma globulins
ARG	Aggregated rabbit gamma globulins
BSA	Bovine serum albumin
Tween 20	Polyoxyethylene-sorbitan monolaurate
iv	Intravenous injection
im	Intra muscular injections
PEG	Polyethylene glycol
OD	Optical Density
NZW	New Zealand White (Rabbit)
PBS	Phosphate buffered saline
VBS	Veronal buffered saline
CHD	Coronary heart disease
MCi	Millicurie
MJ	Megajoule
Mmol	Millimole
ng	Nanogram 1×10^{-9}g
µg	Microgram 1×10^{-6}g
mg	Milligram 1×10^{-3}g
g	gram
kg	Kilogram 1×10^{3}g
µl	Microlitre 1×10^{-6}ℓ
ml	Millilitre 1×10^{-3}ℓ
ℓ	Litre
DSM	Dried Skimmed Milk

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CHAPTER 1

GENERAL INTRODUCTION

1. General Introduction

The immune system is a complex network of interacting cells and antibodies functioning to maintain homeostasis by protecting the body from invading foreign material.

When the immune system is stimulated to produce a response which is harmful to the tissues or disrupts the physiology of the host the term 'allergy' or 'hypersensitivity' may be used.

Adverse reactions to food are numerous and can only be described as food allergy when an immunological reaction occurs after the ingestion of specific foods *(Bleuminck, 1979)*.

Allergy to food proteins can occur at any age but it is more common in the infant. The major food causing adverse reactions in infants is cows milk and this is often the first sign of an allergic disposition which later in life may lead to eczema, asthma or other atopic diseases.

The type of immune response an individual produces to a particular dietary protein will depend on the quantity of antigenic protein entering the circulation, previous encounters with the protein, the age of first exposure to the protein and the presence or absence of maternal antigodies.

The following sections expand on these factors by examining the handling of dietary antigen by the intestine, acquisition of passive immunity and infant feeding and finally the immune mechanisms involved in tolerance to ingested antigens.

1.1 Antigen Handling by the Gut

The major function of the intestine is to digest food and absorb nutrients, the digestive activity in the lumen is largely provided by the enzymes secreted by the pancreas. This results in the mucosal cells of the intestine being continually bathed in luminel fluid which contains a mixture of undigested and partially digested food, microbial antigens and possible infective organisms.

A second function of the gut is to protect the body from the invasion of antigenic macromolecules and pathogenic organisms. The largest potentially antigenic load to which the body is continually subjected involves the food ingested in everyday life. The gastrointestinal tract exhibits several defense mechanisms to prevent the

entry of possible antigens into the circulation.

The mucus covered epethelial surface, together with the lamina propia, provide not only a physical barrier but also an immunological barrier against antigenic material.

1.1.1 Physical Factors Preventing Macromolecular Absorbtion

The evidence that the indigenous intestinal flora is able to protect the gut mucosa from antigen invasion has largely come from using germ-free animals. In the germ-free state the intestinal wall is considerably thinner, less cellular *(Gordan,1960; Gordan & Pesti, 1971)* and the normal development of the intestinal epethelia is prevented *(Abrams, 1977; Abrams et al., 1963; Khoury et al., 1969)*. The cellularity and volume of the lamina propria is also diminished *(Hereghen, 1965)*.

As well as encouraging the normal development of the gut morphology, the resident intestinal flora has been shown to limit the growth of pathological bacteria within the intestinal lumen. This may be due to competition between the indigenous and pathological bacteria, either for substrates or by alteration in the microenvironment for optimal growth *(Freter, 1962)*. This limitation on the growth of pathogenic bacteria leads to a reduction in the release of antigenic substances within the intestinal lumen. This subject has been reviewed by *Simon & Gorbach (1981)*.

The epethelial cells of the gut wall are also protected from the lumenal contents by a mucus coat lining which has been proposed as an antiviral and antibacterial substrate. The glycoproteins and glycolipids known as mucins present in the mucus may bind directly with the organisms or its toxin and thereby inactivate it *(Forstner, 1978; Stomeck & Harrold, 1974)*. Several studies suggest that the digestive effect of gastric acid and pepsin may prevent the migration of micro-organisms, toxins and other ingested antigenic material into the digestive tract, which is possibly assisted by peristalsis *(Draser et al., 1969; McClelland, 1979)*.

1.1.2 Immunological Factors Preventing Macromolecular Absorbtion

The lymphoid tissue of the intestine is termed the gut associated lymphoid tissue (G.A.L.T.). The GALT is comprised of several lymphoid populations which can be identified on the basis of anatomical and

morphological considerations. These populations include collections of organized lymphoid nodules called Peyers Patches (P.P.), scattered lymphoid follicles and small aggregates of follicles which line the intestine, also the loose connective tissue of the lamina propria is populated with lymphocytes and plasma cells. This lymphoid tissue enables the intestine to mount an immunological response to antigenic challenge *(Kagnoff, 1981)*.

1.1.2.i Peyers Patches

Peyers Patches are the major lymphoid population of the GALT involved in the initiation and expression of mucosal immunity. They are groups of subepethelial lymphoid follicles located throughout the small intestine. Light microscopic studies have revealed that regardless of the number of follicles present in a patch, each patch is only one cell thick, thus preserving the intimate relationship between the follicle and overlying epethelium *(Faulk et al., 1970)*. Each patch contains three distinct areas - the dome, the follicle and the 'thymus dependent area', in addition to the villi of the conventional bowl mucosa *(Wakesman, 1973)*. T. and B lymphocytes appear to be compartmentalised in the patch; B lymphocytes tend to be located in the dome region of the patch, whereas thymic cells are located in the thymus dependent area *(Wakesman, 1973)*. Values of between eleven and forty percent of the lymphocyte population of the adult Peyers Patch have been found to be T-lymphocytes, whereas B lymphocytes make up forty to seventy percent of the population *(McWilliams et al., 1974; Raff et al., 1971)*.

Craig and Cebra (1971) identified an enriched source of intestinal antibody producing cell precursors as a subset of B lymphocytes which migrate and proliferate in the lamina propria *(Craig & Cebra, 1975)*. The migration of the lymphocytes to the lamina propria probably involves the cooperation of T cells and macrophages *(Elson et al., 1970; Kawanishi et al., 1982; Kawanishi et al., 1983)*.

Gut antigens appear to be sampled by the Peyers Patch, which leads to an increase in the appearance of specific antibody producing cells in the lamina propria *(Cebra et al., 1977; Husbands & Gowan, 1978; Keren et al., 1978)*. The sampling of the gut antigens may be a function of the specialized cells covering the follicles, described by *Owen and Jones (1974)* and termed 'M cells'.

1.1.2.ii Immunoglobulin A

Immunoglobulin A is the principal immunoglobulin in secretions of the gastrointestinal tract, as well as other external secretions such as tears, saliva, and secretions of the respiratory tract *(Tomasi & Grey, 1972)*.

The antibody response of the intestinal mucosa is therefore mediated largely by lgA which is produced locally in the lamina propria by plasma cells derived from the B lymphocytes located in the Peyers Patch *(Lamm, 1976; Pierce & Gowans, 1975)*.

The majority of lgA in external secretions differs from lgA in the serum and it is referred to as secretory lgA (slgA) *(Tomasi et al., 1965)*.

The molecule is a dimer consisting of two monomer subunits *(Cebra & Robbins, 1966)*, plus two extra polypeptide chains, one is a glycoprotein synthesised and secreted by glandular epethelial cells and is called the secretory component, the second polypeptide chain is termed the J-chain which joins together the two lgA monomer subunits *(Bienstock, 1981; Tomasi & Grey, 1972)*. Polymeric lgA accounts for over 90% of jejunal lgA which is mostly bound to a secretory component, although the secretory component is also present in the free form *(Jonard et al., 1984)*.

The presence of the secretory component increases the resistance of dimeric slgA against proteolysis and also possibly is important in the transport of slgA across the epethelial cell *(Steward, 1971; Lindh, 1975; Valnes et al., 1984)*.

Although the exact function of slgA is not known, evidence suggests that it is the major class of immunoglobulin involved in protecting the gut from the invasion of antigenic substances. slgA has been shown to prevent antigen absorbtion in the gut and respiratory tract by the antigen binding properties of the immunoglobulin *(Andre et al., 1974; Walker et al., 1974; Stokes et al., 1975)*. *Williams and Gibbons (1972)* found that slgA can prevent bacteria from adhering to the epethelial cell surface, thus preventing bacterial colonization, and *Walker et al., (1972A and 1975C)* have shown that slgA can inhibit the adherence of other antigenic substances onto intestinal cellular surfaces.

Because of these mechanical and immunological defence mechanisms of the gastrointestinal tract, it has been assumed that the intestine is

impermeable to antigenic material. However, there is now substantial evidence to show that antigenically active molecules can penetrate the epethelium, not in sufficient quantities to be nutritionally important, but in quantities of immunological importance.

1.2 Antigen Absorbtion by the Gut

In 1911, *Wells* observed that sufficient quantities of egg protein could be absorbed by the guinea pig to cause anaphylaxis, which was confirmed in subsequent studies *(Alexander et al., 1936; Bernstein & Ovary, 1968)*. *Danforth and Moore (1959)* also noted that insulin injected into isolated loops of adult small intestine produced a hypoglycaemic response.

In man, the detection of circulating antibodies and immune complexes to food proteins has provided evidence that macromolecular protein crosses the intestine and enters the circulation *(Peterson & Good, 1965; Paganelli et al., 1979)*.

To substantiate these physiological observations above, the election dense protein, horse radish peroxidase (HRP) has been used in electron-micrographic studies to monitor the passage of intact HRP across the small intestine into the portal venous and mesenteric lymph flows *(Cornell et al., 1971; Warshaw et al., 1971)*.

1.2.1 Mechanisms of Antigen Absorbtion

Isolated gut sac preparations were used to investigate the process by which antigen is absorbed across the intestine. Studies using HRP revealed that the molecule is transported across the epethelial cell by a mechanism resembling the endocytosis reported in neonatal animals and similarly to other endocytotic processes, energy is required *(Walker et al., 1972B)*.

Williams and Hemmings (1978) showed the transport of ^{125}I labelled bovine lgG and ferritin across the intestine of 100 day old rats. They suggest that the macromolecules enter the cell by a non-selective diffusion process, possibly with the assistance of the glycocalyx. Another possibility is the involvement of the M cell which overlies the GALT, this route of antigen absorbtion would result in the access of antigens to the lymphocytes in the GALT *(Owen, 1977)*.

The quantity of macromolecular protein that is absorbed from the gastrointestinal tract has not been confirmed. *Hemmings & Williams (1978)*

have reported that up to 40% of a dose of bovine lgG and α gliadin given to rats is absorbed in the form of large molecular breakdown products. However, one, explanation for this high value is that radiolabelled fragments of macromolecules have been observed to bind to host proteins such as albumin. The labelled amino acids and small peptides therefore increase in apparent molecular size and become TCA precipitable *(Udall, Bloch et al., 1981)*.

A more realistic value is perhaps that up to 2% of food protein is absorbed in this way *(Warshaw et al., 1974)*. Absorbtion into the portal blood and lymphatics appears to be independent. In the rat, 0.76% has been found to enter the lymphatics while 1.11% of the total absorbed enters the portal blood stream *(Warshaw & Walker, 1974)*.

Despite this continuous antigenic challenge, most adults have only low levels of circulating antibodies to common dietary proteins and show no ill effects from a limited absorbtion of macromolecules. Therefore, it seems likely in cases of allergy or hypersensitivity that a defect occurs allowing excessive quantities of antigenic material to enter the circulation and result in clinical disease.

1.2.2 Pathological Absorbtion of Antigen

Factors that may cause excessive absorbtion of macromolecules include:

i) Decreased intralumenal digestion - possibly due to a decrease in gastric acid production or pancreatic insufficiency *(Walker et al., 1975A)*.

ii) Disruption of the mucosal barrier - this may occur in certain situations of 'stress' and disease. Crohns enterocolitis, ulcerative colitis, coeliac disease and diarrhoea in infants have all been associated with an increase in circulating serum antibodies *(Taylor & Truelove, 1961; Gruskay & Cooke, 1955)*.
Rhodes and Karnovsky (1971) have demonstrated a loss of barrier function after surgery and *Worthington and Boatman (1974)* and *Worthington, Boatman and Kenny (1974)* have shown a deterioration of the apical cell junction in protein-calorie malnutrition allowing an increase in macromolecules crossing the epethelial cell lining of

the gut. Malnutrition has also been found to cause immaturity of the epethelial cell *(Chandra, 1975)*. Other conditions which may damage the mucosal epethelial barrier include infection, drug therapy and radiation treatment.

iii) Lysosomal dysfunction - This would result in a decrease in intracellular proteolysis. *Worthington and Styrctuck (1976)* have suggested a lysosomal deficiency in protein calorie malnutrition and the immature cells of the neonate may also be deficient in lysosomal activity *(Walker & Isselbacher, 1974)*.

iv) Local Antibody Deficiency (Refer to Section 1.1.2.ii) Local immunoglobulin is important in the handling of antigen in the gut and selective lgA deficiency has been found to result in a high incidence of circulating anti-food antibodies *(Buckley & Dees, 1969; Cunningham-Rundles et al., 1978)*.

v) The neonatal gut - The immature gut of the neonate is more permeable to macromolecules than the adult or older child *(Rothberg, 1969; Easthman et al., 1978)*. This may be due to deficiency of slgA *(Taylor et al., 1973)*, immature epethelial cells, or lack of lysosomal activity. A recent study has demonstrated that there is decreased intestinal proteolytic activity in newborn rabbits compared to weaned animals *(Udall et al., 1984)*. Thus, during this vulnerable period the neonate receives protection from the invasion of foreign protein by the presence of maternal antibodies.

1.3 Transmission of Maternal Antibodies

From the moment of birth the young mammal must be equipped with a highly effective immunological defence system to protect itself against a wide range of pathogenic agents in the environment. However, in mammals the placenta and foetal membranes in conjunction with maternal immune responses present an effective barrier in protecting the foetus from infective organisms, therefore few of the antigenic stimuli experienced by the mother are likely to be experienced by the foetus. Thus, prior to birth the necessary education of the lymphoid system

needed to combat infection has not occurred and only commences after birth when the newborn mammal is exposed to the majority of antigens for the first time. During the neonatal period, the presence of protective maternal antibodies are vital while the immune system of the newborn is maturing.

The time at which the animal receives maternal antibodies has been well reviewed by *Brambell (1970)* and is summarized in Table 1.1. Transmission of passive immunity may take place either before birth, via the yolk sac or placenta, and/or after birth from the maternal milk or colostrum (Table 1.2). The calf, sheep, goat, horse and pig do not receive any maternal antibodies in utero and therefore rely entirely on the antibody rich colostrum received immediately after birth. The maternal antibodies in the colostrum are rapidly transmitted to the circulation by a non-selective uptake process *(Leary & Lecce, 1979)*.

In contrast, the human infant and the young of monkeys, rabbits and guinea pigs are born well equipped with passive immunity. At birth, the maternal antibodies in the young of these species are at a concentration equal to or even greater than the concentration in the maternal circulation. The major colostrum immunoglobulin of these species is secretory IgA *(Beer et al., 1974)*, which cannot readily be transported across the intestinal barrier. It appears to be capable of binding to enterocytes but does not become internalised or transported *(Nagura et al., 1978)*.

The rat and mouse form an intermediate group, passive immunity being received both before and after birth in the young of these species. However, although a significant amount of maternal antibodies are transmitted across the foetal membranes in utero, the majority of maternal antibodies are thought to be received after birth from the colostrum and milk. Unlike the ruminant, horse and pig, postnatal transmission of maternal antibodies is not limited to a few hours after birth, but continues for the greater part of the lactational period *(Brambell & Halliday, 1956)*.

Selective transmission of immunoglobulins occurs in the placenta of the primate *(Halliday & Kekwick (1960) & Wild, 1981)* and at the intestinal wall in the rat and mouse *(Bangham et al., 1956; Quinliven, 1967)*.

TABLE 1.1

Time of Transmission of IgG from Mother to Foetal and Newborn Mammals

Species	Prenatal	Postnatal	Duration of Postnatal Transfer
Ox, goat sheep	O	+++	24 hours
Pig	O	+++	24 - 36 hours
Horse	O	+++	24 - 36 hours
Wallaby	O	+++	180 days
Dog, Cat	+	++	1 - 2 days
Hedgehog	+	++	40 days
Mouse	+	++	17 days
Rat	+	++	21 days
Guinea Pig	+++	O	
Rabbit	+++	O	
Grey Squirrel	+++	Unknown	
Man, Rhesus Monkey	+++	O	

(Adapted from *Billington & Wild, 1979)*

O - No transmission of maternal antibodies

\+ - Represents approximately 1/3 of total amount of passive immunity acquired

TABLE 1.2

Route of Transmission of IgG from Mother to Foetal or Newborn Mammals

Species	Route	
	Prenatal	Postnatal
Ox, goat sheep	None	Gut
Pig	None	Gut
Horse	None	Gut
Dog, Cat	None	Gut
Hedgehog	None	Gut
Mouse	Yolk sac	Gut
Rat	Yolk sac	Gut
Guinea Pig	Yolk sac	None
Rabbit	Yolk sac	None
Man, Rhesus Monkey	Chorio-allantoic Placenta	None

(Adapted from *Brambell (1970)*
Billington & Wild (1979))

1.3.1 The Mechanism of Selective Transmission of Maternal Antibodies

Transmission of maternal antibodies is selective before birth in the rabbit, guinea pig, monkey and man and after birth in the rat and mouse (Table 1.3).

Selective transmission in the ungulate is restricted to the secretory follicles of the mammary gland. Antibodies pass from the maternal plasma to the lacteal secretions causing a decline in the maternal serum gamma globulins at the time of colostrum formation *(Dixon et al., 1969)*. The mammary gland selectively concentrates lgG1 in the cow *(Morgan et al, 1981)* and lgG2 in the sow. Thus 80% of total colostrum immunoglobulin is lgG in these species *(Porter, 1979)*. Therefore, although immunoglobulins of the lgG, lgM and lgA class are absorbed non-selectively (with the exception of 11s lgA in the pig - see *Porter, 1976)* by the piglet and calf, the majority of antibody received from the colostrum will be immunoglobulin G *(Billington & Wild, 1979)*.

Primates are the only class of mammal which receives maternal antibodies exclusively by placental transmission, which is selective for immunoglobulin G. It is believed that the majority of immunoglobulin is transmitted during the second half of the third trimester *(Valquist et al., 1950; Gitlin et al., 1964; Balfour & Jones 1976)*. The placenta of the rabbit and human are of a similar type, therefore it was believed that immunity was transmitted by the same route in both species. However it is now well established that in rabbits maternal antibodies pass by way of the uterine lumen and foetal yolk sac *(Brambell et al., 1949)*. Only antibodies of the lgG class are transported intact across the lumen chorio-allantoic placenta and suckling rat and mouse gut to any appreciable extent and largely to the exclusion of other serum proteins *(Brambell, 1970)*. The situation in the rabbit is more complex, since there is evidence that lgM may also be transported but probably not normally by the same route as lgG *(Shek & Dubiski, 1975)*.

Both the yolk sac and neonatal intestine have been used to study the process of selective uptake of maternal antibodies. Electron-microscopy has shown striking similarities between the enterocyte morphology of the human placenta, neonatal rat intestine and rabbit yolk sac which all manifest the structure of an active pinocytotic cell *(Clark, 1959; Wissig & Graney, 1968)*.

In 1966 *Brambell* proposed the receptor hypothesis to account for

TABLE 1.3

Site of Selectivity of Transmission of Maternal Antibodies from Mother to Young *(Brambell, 1970)*

Species	Route	Site of Selectivity
Horse, Cow Sheep, Pig	Colostral-Intestinal	Mammary Gland
Rat, Mouse	Yolk sac (minor route) & colostral-intestinal	Intestine
Rabbit	Yolk sac	Yolk sac
Man, Monkey	Placental	Placenta

the selective transmission of antibodies, which is represented in a digaramatic form in Figure 1.1. The hypothesis suggests that antibodies enter the enterocyte by non-selective pinocytosis. Inside the pinocytotic vacuole, the immunoglobulin molecule is able to bind to specific receptors which protect them from lysosomal degredation. The receptors favour homologous lgG which binds to the receptor via the Fc portion of the molecule.

Subsequent studies suggested that antibodies were selected from other proteins by Fc receptors at the base of the apical cell surface indicating that selection is extra-cellular rather than intracellular *Rodewald, 1973)*.

The selection of antibody of the lgG class is believed to occur in the proximal part of the intestine. Rat γ globulin was found to bind to the isolated microvillus membrane from rat neonatal jejunum but not to neonatal ileum *(Jones & Waldman, 1972)*. A number of other studies have since confirmed that lgG selectively binds to the proximal region of the small intestine *(Borthistle et al., 1977; Morris, 1975; Morris & Morris, 1976)*.

The receptor hypothesis originally proposed by Brambell has since been modified to allow the observation made by *Rodewald* on the neonatal rat intestine and the similar observations made by *Wild (1976)* on the selective transmission of antibodies across the rabbit yolk sac. Both authors conclude that lgG destined for transport across the absorbtive cell is first selectively taken up into a distinct class of endocytic vesicles termed 'coated vesicles' *(Wild, 1980)* via specific Fc receptors on the enterocytes of the suckling rodent gut, possibly located in the glycocalyx and on the rabbit yolk sac endoderm. Thus allowing segregation of lgG from other protein molecules to occur before entry into the cell. These coated vesicles were suggested to be protected from lysosomal degredation allowing their contents to be released intact by reverse pinocytosis. However, in the case of the rat intestine, there is now evidence to show that lgG is transported across the cell as an lgG-receptor complex. The continued binding of lgG to the vesicle membrane appears to be required for successful transfer as a certain amount of apparently non-selective protein uptake also occurs by endocytosis, but these proteins were not released at the lateral surface of the cell *(Abrahamson & Rodewald, 1981)*.

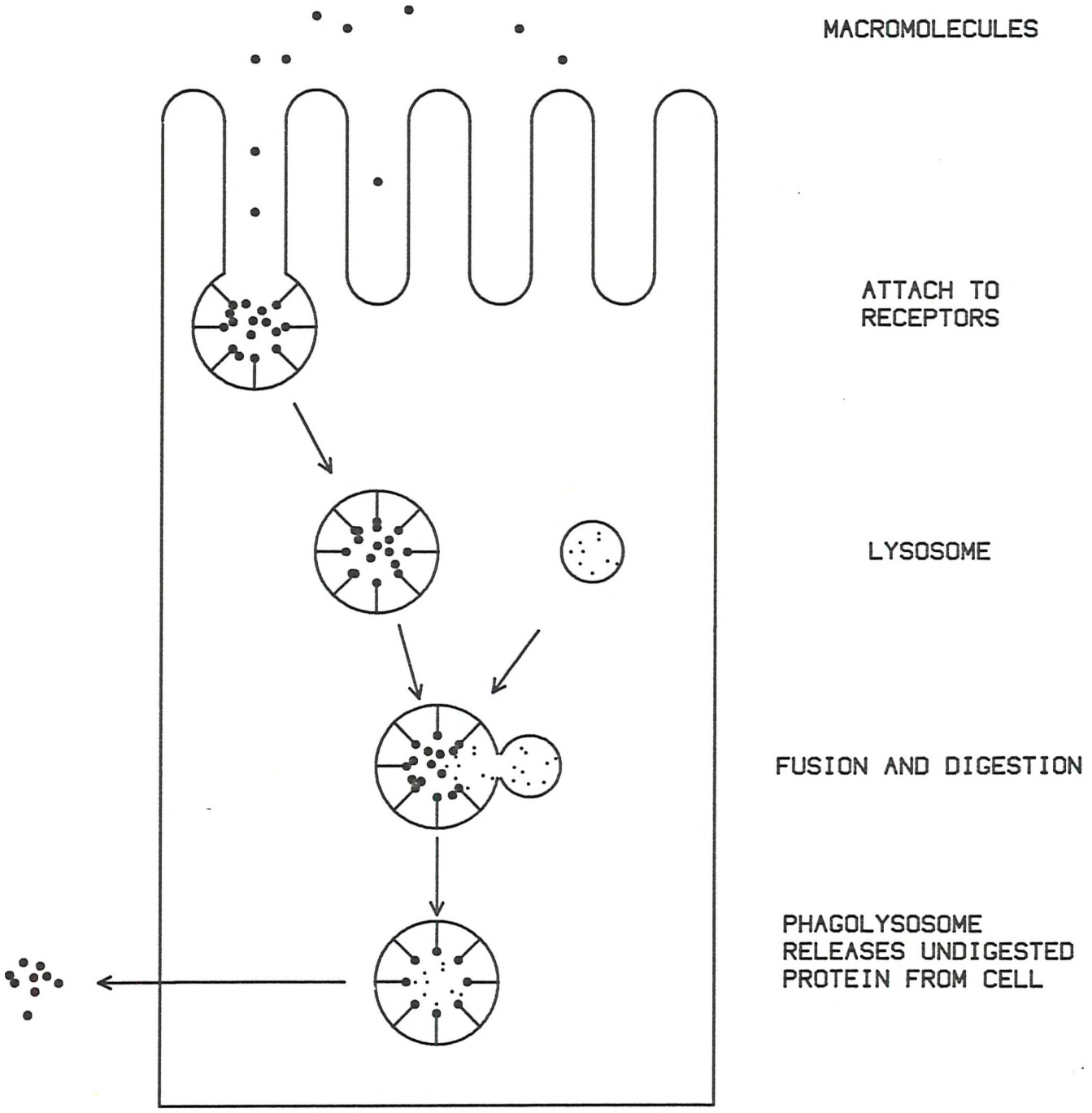


Figure 1.1 MECHANISM OF GLOBULIN TRANSMISSION BY THE CELL (Brambell, 1966)

1.3.2 Gut Closure

The termination of postnatal absorbtion of maternal antibodies is referred to as 'gut closure'. This process occurs at approximately 24 to 36 hours after birth in the pig, calf, horse and sheep and may depend on some factor in the colostrum *(Payne & Marsh 1962; Lecce & Morgan 1962; Lecce et al., 1964)*. Most of the passive immunity is acquired in the first few hours after birth. In the pig, maximum absorbtion occurs 4 - 12 hours after birth which appears to be independent of intestinal proteolytic activity *(Weström et al., 1984)*. The mechanism of gut closure may depend on a number of factors occurring in the neonatal ungulate soon after birth such as exhaustion of pinocytotic capability, cell replacement and maturation of proteolytic mechanisms *(Porter 1979)*.

The process of gut closure appears to be via a different mechanism in the rat and mouse. The gut of the neonatal rat and mouse absorb antibodies for 21 days and 17 days respectively *(Halliday, 1956)*. Before there is complete termination of selective antibody transmission there is a period of decreased absorbtion which occurs at 18 - 21 days in the rat and 14 - 17 days in the mouse. During this period of morphological maturation of the intestinal epethelial cells Fc receptors fail to become expressed on the intestinal enterocyte surface. This failure of Fc receptor expression can be precociously induced by the administration of large doses of deoxycorticosterone and cortisone acetate *(Halliday, 1959; Wild, 1981)* resulting in premature gut closure.

The intestine of the human premature infant is more permeable to macromolecules than the adult even though this is not the major route for the acquisition of maternal antibodies. The process of gut closure in man and rabbit appears to be more subtle involving structural and functional maturation of the intestine which decreases but does not completely abolish macromolecular absorbtion.

Studies on near-term foetal monkeys by *Lev & Orlic (1973)* and human foetuses by *Moxey & Trier (1975)* have shown excessive uptake of large molecules by intestinal epethelial cells and describe morphological features of the cells suggesting structural immaturity. Gut closure in the infant has been suggested to occur before birth; *Roberton et al. (1982)* found that macromolecular absorbtion in the premature neonate decreased with increased gestational age, particularly after 33 weeks

gestation. Other studies on rabbits have found a progressive decrease in antigen absorbtion from birth, particularly during the first week post-partum *(Udall, Pang et al., 1981)*. This evidence, together with the observation that young infants have higher circulating antibody titres than adults or older children *(Gunther et al., 1962; Rothberg & Farr, 1965; Korenblat et al., 1968)* indicates that the gut of the neonate is more permeable than that of the adult. This may be the result of a number of factors such as a deficiency in secretory antibodies *(Soothill, 1976)*; an increase in affinity of antigen to the mucosal surface; enhanced pinocytosis or a decrease in intercellular or intracellular proteolysis of the immature gut *(Walker 1975)*.

During this period of enhanced macromolecular absorbtion the immunological as well as the nutritional consequences of the infant's diet is important.

1.4 Infant Feeding

Human colostrum and milk contains a variety of factors to protect the newborn infant from infection - these include T and B lymphocytes, macrophages and other mononuclear cells *(Hanson & Winberg, 1972)*. Breast milk also contains antibodies against micro-organisms and other foreign proteins. In contrast to the serum in which IgG is the type of immunoglobulin which predominates the major antibody in the milk is secretory IgA *(McClelland, 1982)*.

A close correlation exists between intestinal antigenic stimulation and antibodies in the mammary secretion, this may be due to the selective transfer of lymphocytes from the gastrointestinal tract to the mammary gland via the lymphatics *(Goldblum et al., 1975)*. This close relationship observed between the lymphoid tissue of the human mammary gland is supported by the constant demonstration of sIgA in human milk against cows milk proteins *(McClelland & McDonald, 1976)*.

The importance of sIgA in protecting the intestine from the invasion of antigenic macromolecules has been outlined in Section 1.1.2.ii. Thus the sIgA present in breast milk produced against a wide variety of micro-organisms and dietary proteins is likely to help protect the gut of the newborn infant from excessive absorbtion and therefore sensitization to environmental antigens. The importance of sIgA in breast milk may be demonstrated by the increased incidence of cows milk allergy *(Busseret, 1978)*, asthma *(Kaufman & Frick, 1981)* and eczema *(Mathew et al., 1977)* which has been observed in bottle compared to breast fed

infants.

IgA in breast milk may bind to the mucosa of the infants intestine which may involve the leukocytes present in the milk.

Leukocytes are composed primarily of lipid-laden neutrophils and macrophages which are phagocytotic *(Johnson et al., 1980);* they also contain large amounts of sIgA which may be transported to the newborn infant *(Pittard et al., 1977)*. A proposed hypothesis for breast milk leukocytes is that they attach themselves to the mucosa of the infant's digestive tract during the phagocytosis of colonizing micro-organisms. The release of sIgA from the leukocyte seems to depend on certain membrane stimuli indicating that sIgA would only be released after binding to mucosal epethelial cells *(Weaver et al., 1984)*. Maternal leukocytes have been found to be capable of penetrating the epethelial lining of the gastrointestinal tract of the newborn *(Schnorr & Pearson, 1984)*.

Breast milk sIgA has also been shown to bind to the epethelial cells of the neonatal rat jejunum, but unlike IgG it is not internalized and translocated across the cell *(Nagura et al., 1978)*.

It is likely that when an alternative food to breast milk is fed to the neonate the absence of sIgA provided by the breast milk, together with the ingestion of a foreign protein increases the risk of specific systemic sensitization, even though in some cases sensitization of the infant may occur from antigen present in the maternal milk *(Gerrard, 1979)*.

The best combination appears to be the presence of circulating maternal antibodies, together with the protective effect of breast feeding for inducing a state of systemic tolerance to dietary proteins. This is probably why the immune responses of infants fed cows milk have been found to be lower in those infants who had initially been fed breast milk *(Kletter et al., 1971)*.

The significance of antibodies to food proteins in the serum is debatable. Increased levels of specific IgG antibodies may indicate food intolerance or merely represent the normal response for removing foreign antigenic protein from the body. The following sections discuss the mechanisms of systemic unresponsiveness which is observed in the majority of the population after the ingestion of dietary proteins and the possible relationship between circulating antibodies to food proteins and atherosclerosis.

1.5 Immune Tolerance to Ingested Antigens

In various animal models with different antigens and assays, deposition of antigen in the gut may, under certain circumstances, produce a local immune response simultaneously with systemic tolerance. This form of split tolerance probably represents the appropriate response to common, but otherwise harmless, ingested antigens.

The suppression of antibody formation and delayed type hypersensitivity responses by prior oral administration of antigen has been known for many years *(Wells, 1911; Chase, 1946)*. Subsequent reports in which either cellular or soluble antigens have been fed confirm that antigen administration by the intestinal route can alter the immune response in extra intestinal sites. *Thomas and Parrott (1974)* found a reduced antibody response to parenterally administered BSA when preceded by a period of antigen feeding, and *Kagnoff (1978)* observed a depression of delayed type hypersensitivity response in mice to sheep red blood cells after prior feeding of the antigen. However, the precise mechanism by which this form of tolerance is induced has not yet been fully explained.

A number of papers have implicated the importance of suppressor T cells after feeding both soluble and cellular antigens *(Ngan & Kind, 1978; Mattingly & Waksman, 1978)*. It appears that hyporesponsiveness can be produced in B cells after the oral administration of antigen but that the unresponsiveness observed in these cells is of shorter duration than is manifested by the T cell *(Vives et al., 1980)*. *Asherson et al. (1977)* have demonstrated the presence of T and B cells in mice after feeding the mice contact sensitizing agents, and both types of suppressor cell appear to be important in the resulting unresponsiveness, which can be enhanced by feeding B cell mitogens *(Newby et al., 1980)*.

Two distinct non-immunoglobulin suppressor factors have been identified which are likely to be T cell derived. The first factor enhanced the suppressive activity of T cells and the second both helped and induced feedback suppression *(Mattingly et al., 1980)*.

Presentation of the antigen to the Peyers Patch appears to be an important stage in the induction of oral tolerance, and this may account for differences in which tolerance is induced against a particular antigen for example soluble or cellular antigens. Suppressor T cells are apparent in the Peyers Patch after feeding lower doses of antigen than is required to induce suppressor cells in the spleen *(Ngan & Kind, 1978)*.

The observation that systemic hyporesponsiveness was accompanied by an increase in slgA copro-antibody indicated that perhaps reduced enteric absorbtion of antigen could account for the systemic unresponsiveness *(Andre et al., 1974)*.

Swarbrick et al (1979) found that prior feeding of antigen reduced its subsequent absorbtion into the circulation and *Challacombe and Tomasi (1980)* observed that intragastric administration of a soluble or particulate antigen resulted in the concurrent induction of salivary antibodies and systemic suppression. *Andre et al (1975)* have found that the hyporesponsiveness produced in this way can be transferred in the serum to untolerized recipients indicating the presence of a soluble tolerogen. They suggest immune complexes containing lgA as the antibody component are the tolerogen and may function by paralysing B cells.

The central role of the apparent paradox of mucosal immunity and systemic tolerance may be played by the Peyers Patch. A specific class of helper T cells have been identified in the Peyers Patch *(Elson et al., 1979)*. They appeared 24 hours after enteric administration of ovalbumin and simultaneously or immediately afterwards, lgG specific suppressor T cells made their appearance *(Richman et al., 1981)*. These specific T cells have been found to migrate from the GALT to the spleen shortly after contact with antigen and have been referred to as T cell suppressor inducer cells. The cell interacts with splenic lymphocytes to induce the formation of an effector T suppressor cell *(Mattingly 1984)*. Suppressor T cells have been found after exposing foetal mice to deaggregated human gamma globulin (dHGG) transplacentally, and after exposing neonatal mice to dHGG via the colostrum. However, tolerance was only generated by the administration of higher doses of dHGG in utero and tolerance was not observed in those mice receiving dHGG via the colostrum, which was possibly due to the smaller quantity of antigen received by the neonate *(Fazekas de St Groth et al., 1984)*. Tolerance was suggested to occur by the suppressor T cells inactivating B cells and possibly T helper cells. These specific suppressor T cells in the GALT may regulate the immune response engendered by the encounter with a dietary antigen and explain the paradox of mucosal immunity and systemic tolerance. The ability of an antigen to produce systemic tolerance may depend on the ability of an antigen to stimulate suppressor

or helper T cells. An antigen usually has both helper and suppressor T cell determinants and stimulation of specific T cells may depend on the concentration of the antigen and the possible competition of suppressor and helper T cells for antigenic determinants *(Kölsh, 1984)*.

However, the precise mechanism responsible for inducing tolerance in the humoral and cell mediated immune system remains contraversial.

Cell mediated but not humoral tolerance can be transferred by the serum *(Strobel et al., 1983)*, and a lower dose of ovalbumin is needed to suppress cell mediated immune responses than antibody responses *(Mowat et al., 1982)*, indicating that more than one regulatory factor is involved in controlling the two limbs of the immune system.

The liver, through which gastrically administered antigens pass, may play a critical role in systemic unresponsiveness by removing antigens from the portal blood stream *(Thomas et al., 1976)*. This function has been ascribed to the Kupffer cell, which can be regarded as the liver macrophage. Its role in the hyporesponsive state may lie in its ability to sequester antigen from the circulation and also perhaps through the induction of specific tolerance via the depression of T cell responses, either directly or by secretion of inhibitory factors *(Richman et al., 1979)*.

In a feeding study in rabbits it was fortuitously found that the offspring of dams fed the same protein as that in the weanling diet developed lower circulating antibodies to that protein when compared to similar weanling rabbits bred from dams fed a control diet *(Pathirana et al., 1979)*. Concurrently with the observation that these weanling rabbits showed specific systemic tolerance to soy protein, they were also found to have less arterial disease than the control group, which manifested higher antibody titres.

This finding was substantiated by a further experiment in which circulating anti-soya antibodies could be correlated with the development of aortic atheroma *(Gallagher et al., 1982)*.

1.6 The Immunological Theory of Atherosclerosis

There is a certain amount of evidence to suggest that immunological mechanisms play a part in the development of atherosclerosis.

One of the main areas of evidence for an immunological basis for human atherosclerosis comes from the observation that very rapid and severe arterial disease often develops in the recipients of organ transplants *(Thompson, 1969)*.

Post mortem examinations of heart transplant patients reveal extensive coronary artery disease with both poliferative and intimal lesions *(Griepp et al., 1977)*. Similar complications are observed in kidney transplant patients. Renal artery stenosis is a well recognised problem of renal transplantation caused by occlusive arterial disease. This frequently leads to progressive hypertension and loss of graft function *(Mollenkopf et al., 1977;)*

The severe atherosclerosis following organ transplants has also been observed in animal models *(Minick et al., 1974)*. *Alonso et al. (1977)* have induced arterial intimal thickening in rabbits by cardiac allografts and with the addition of cholesterol supplemented diets many of the lesions transformed to cholesterol filled atheromatious plaques. These plaques were similar to the lesions seen in human atherosclerosis. *Lurie et al. (1981)* has suggested that the vascular lesions may be caused by cytotoxic antibodies against the allografts.

Further evidence for the involvement of immune mechanisms in the development of atherosclerosis has come from producing serum sickness in experimental animals. Several groups have reported that aortic atherosclerosis can be hastened by the injection of horse serum albumin or bovine serum albumin *(Minick et al., 1966; Lamberson & Fritze, 1974)*. Similarly in primates the injection of an antigen has produced accelerated atheroma development in animals fed a mildly atherogenic diet compared to animals fed the atherogenic diet alone *(Howard et al., 1971; Stills et al., 1983)*. *Stills et al. (1983)* found that this form of atherosclerosis was more pronounced in the proximal coronary vessels and coronary artery compared with cholesterol induced atherosclerosis. The lesions consisted primarily of foamy intimal cells with granulocytes attached to and penetrating into the lamina of the artery.

These observations suggest that immunological response produced by either serum sickness or graft rejection may act in synergy with hypercholesterolaemia to cause the rapid development of atherosclerosis.

Hardin et al. (1973) has reported that acquired arteriosclerotic lesions produced by serum sickness can predispose to later lipid accumulations and evolve as atherosclerosis. Immune complex deposition has been implicated as the cause of the intimal endothelial damage resulting in arteriosclerotic lesions *(Sharma & Geer, 1977)*.

The fate of immune complexes depends on the physical size of the complex. This is determined by the relative proportions of antibody, antigen and the affinity of the two components *(Devey et al., 1982; Mannick, 1982)*. Large immune complexes are normally formed in antibody excess and these are rapidly removed from the circulation primarily by the Kupffer cells of the liver *(Haakenstadt & Mannik, 1974)*. Complexes formed in antigen excess are usually small and remain in the circulation for a longer period of time before becoming trapped in capillaries of the lung, renal glomeruli or choroid plexus. It is the complexes of intermediate size which are formed in antigen and antibody equivalence or slight antigen excess that have been found to become localized in vessel walls *(Cochrane & Hawkins, 1968)*.

Immune complexes have been implicated as the cause of vasculitis in many disease states. These include rheumatoid vasculitis, systemic lupus erythematosis and various forms of glomerulonephritis, thus affecting medium sized arteries, arterioles and capillaries *(WHO Scientific Group, 1977; Theofilopoulos & Dixon, 1980)*.

Histological and biochemical studies have provided a certain amount of evidence for the localization of immunoglobulin around atherosclerotic plaques. Dense deposits of lgG have been located in such plaques by immunoperoxidase techniques *(Parums & Mitchinson, 1981)* and lgG, lgA, lgM and the complement component C_3 have been identified biochemically *(Hollander et al., 1979)*. The later study suggests that lgG may be synthesised by the artery and may represent an immune response to a continued presence of antigen in the artery. The maintenance of prolonged antibody synthesis by antigen may provide a source of complement fixing antibody-antigen complexes for the mediation of proliferative changes within the artery. However, the mechanism of immune complex deposition and their role in vascular damage can only be speculated upon.

Complexes are not inherently harmful but they may influence the course of atherosclerosis by activating certain immunological and chemical mechanisms. Complexes are able to cause the release of histamine and other vasoactive amines from mast cells and platelets and complexes of intermediate size are known to be able to fix complement *(Reviewed by Haeney, 1982)*. The activation of the complement components C_{3a} and C_{5b} is one of the principal mechanisms by which chemotactic activity for phagocytosis is generated *(Stossel, 1974)*. Immune complex mechanisms are not all mediated through complement. Many cells respond directly to the complexes via surface Fc receptors. Fc receptors have been recognized on monocytes, neutrophils, eosinophils and platelets *(Nydegger, 1979)*. Platelets have been found to react directly with immune complexes resulting in the rapid release of serotonin combined with platelet aggregation *(Pfueller & Leuscher, 1974)*.

Clinical evidence to support the significance of immune mechanisms in the aetiology of atherosclerosis is conflicting. Reports have suggested that the level of soluble circulating immune complexes are higher after myocardial infarction than controls *(Farrel et al., 1977; Fust et al., 1978)*, whereas others were unable to detect any such change *(Gallagher et al., 1982B)*. The observation of high levels of immune complexes in many diseases but no evidence of tissue deposition indicates that the mere presence of immune complexes does not necessarily mean that they are responsible for the disease *(Levinsky, 1981)*. The type of antibody within the complex may be important in determining whether the complex becomes involved in damaging reactions or is merely a mechanism for safely removing circulating antigen. Immune complexes have been observed in healthy adults following the ingestion of food proteins *(Paganelli et al., 1977)*. However, the complexes formed by healthy adults have been found to consist of lgA antibody, whereas those found in atopic food allergic subjects were found to be Clq binding *(Levinsky et al., 1981)*. *Levinsky (1981)* has also observed that the more damaging reactions of some other disease states appear when lgG complexes are produced in preference to lgA complexes.

Thus the normal response to antigen may be the production of systemic and mucosal lgA complexes which rapidly and safely remove the foreign material from the body and it is only when Clq binding complexes are formed that damaging reactions may occur. These complexes appear to be important

in diseases of small vessels and may also be involved in diseases of large vessels, possibly predisposing to atherosclerosis *(Poston, 1979)*.

1.7 Aim of the Research

The aim of the research presented in this thesis is to investigate the immunological response to dietary protein.

The first experiment examines the effect of age on the ability of rabbits to develop specific serum antibodies to dietary proteins, and on the capacity of maternal exposure to suppress this response. Previous studies using rabbits have investigated the immune response to dietary protein in weanling animals *(Pathirana et al., 1981)*. However, this response may be influenced by increased uptake of macromolecular protein in young rabbits *(Udall, Pang et al., 1981)*.

The second experiment investigates the effect of protein source and level on the production of circulating antibodies in rabbits. The capacity of a dietary protein to stimulate the production of circulating antibodies may depend on the amount of protein which crosses the gastrointestinal mucosa and enters the circulation in a potentially antigenic form.

To extend the study of the immune response produced by dietary proteins, the mouse was used in the third experiment, since this species differs from the rabbit in the mechanism by which it acquires maternal antibodies. The mouse receives maternal antibodies both pre- and postnatally, whereas the rabbit acquires all its passive immunity in utero *(Billington & Wild, 1979)*. Therefore, maternal antibodies are likely to be present in the circulation of the young mouse for a longer period of time after birth, compared with the young rabbit. The experiments in this chapter examine the systemic immune response produced in mice, following the ingestion of a specific protein and the possible effects of maternal antibodies on this response.

The first three chapters of this thesis have examined the effect of introducing a novel dietary protein to either rabbits or mice on the production of specific serum antibodies. In the fourth chapter the immune response is examined in humans following the ingestion of a novel dietary protein. The immune response produced by the ingestion of a novel protein is particularly relevant to infant feeding practices involving substitute milks *(May et al., 1982)*.

The hypothesis relating antibodies and circulating immune complexes with increased levels of atherosclerosis *(Gallagher, 1980)* was examined in the final experiment. Systemic tolerance to cows milk protein was

achieved in a group of rabbits by feeding the dams a diet containing milk protein. A high fat cows milk diet was then fed to these animals at weaning, the amount of atherosclerosis in these animals was compared to a group of rabbits bred from dams not exposed to milk protein.

A more detailed introduction accompanied each experimental chapter and the results are also discussed within the chapter. A final discussion is given at the end of the thesis.

CHAPTER 2

MATERIALS AND METHODS

2. Materials and Methods

2.1 An Enzyme-linked Immunosorbant Assay (ELISA) for the Detection of Antigen Specific Serum Antibodies

An enzyme-linked immunosorbant assay based on the method of *Engvall and Perlman (1972)* was chosen for the antibody and antigen assay. The reliability of this technique for diet-induced circulating antibodies has been verified *(Pitts et al., 1983)*.

2.1.1 Method (i)

Polystyrene cuvettes (Gilford Diagnostics, Cleveland, Ohio, U.S.A.) were coated with antigen at a concentration of 10 µg protein per ml dissolved in a carbonate-bicarbonate buffer (50 mM PH 9.6) containing 0.02% (w/v) sodium azide. A 200 µl aliquot of this solution was added to each well, which was incubated overnight at $4^{o}C$ in a humidity chamber. The cuvettes were then washed 5 times, soaking for 30 seconds with each wash (this procedure was used for each wash in the assay). The washing solution was phosphate-buffered saline (PBS) containing 0.05% (w/v) Tween-20 (PBST).

Serum samples were diluted appropriately in PBST and a 200 µl of the diluted serum was added to each well. The cuvettes were then incubated at room temperature for two hours. After a second washing stage, 5 times with PBST, the wells were incubated with a 200 µl of a peroxidase conjugated antimmunoglobulin purchased from Nordic Immunochemical Laboratories, Tilberg, The Netherlands, which had been diluted in PBST 1:1000. After a final incubation for 2 hours at room temperature followed by washing with PBST 5 times, 200 µl of the assay solution was added.

The assay mixture was prepared immediately before use and consisted of a phosphate-citrate buffer (pH 5.0). The buffer contained 24.3 mls citric acid (0.1 M) and 25.7 mls disodium hydrogen phosphate (0.2 M) and 50 mls of distilled water, in this was dissolved 40 mg of O-phenylene diamine (purchased from BDH Chemicals) and 40 µl of 30% (V/V) H_2O_2 solution was added. The colour was left to develop for exactly 15 minutes and then stopped by the addition of 200 µl of a citrate-phosphate buffer (0.3 M pH 2.85).

The optical density of the contents was read at 405 nm by an

enzyme immunoassay manual reader.

A pooled serum sample was run with each assay to check that consistent replication was being achieved.

2.1.2 Method (ii)

Polystyrene microtitre plates comprising of 96 400 µl flat bottomed wells (Dynatech Lt., Billinghurst, Sussex) were coated with 200 µl of a carbonate-bicarbonate buffer (50 mM pH 9.6) containing the dissolved antigen at a concentration of 20 µg protein per ml of buffer and 0.02% (w/v) sodium azide. The plates were incubated overnight in a humidity chamber at 4°C. The microtitre plates were washed 5 times, soaking for 30 seconds with each wash (this procedure was used for each wash in the assay). The washing solution was saline containing 0.05% (w/v) of the detergent Tween 20 (Saline +T).

The serum was diluted with PBST appropriately and 200 µl added to each well. The plates were incubated for 3 hours at room temperature then washed 5 times with saline + T.

A 200 µl aliquot of an antiimmunoglobulin peroxidase conjugate (Nordic Immunochemicals) diluted in PBST 1:1000 was added to each well. After a final incubation step at room temperature for 2 hours, followed by washing 5 times with saline + T 200 µl of the freshly made up assay solution was added. The assay solution contained O-dianisidine HCl (Sigma Chemicals Ltd., Poole, Dorset) (83.3 µg per ml) as the chromogen dissolved in 100 mls of sodium phosphate buffer (0.01M pH 6.0), to this was added 1 ml of 1% hydrogen peroxide solution.

The reaction mixture was left in the wells for exactly 1 hour and the final absorbance was read at 450 nm on an automated dual-wave beam spectrophotometer (MR 580 Dynatech Ltd).

Standardization of the Assay

Daily variations in the activity of the peroxidase enzyme were accounted for by measuring the enzymic activity of the conjugated antibody after each assay.

A 50 µl sample of diluted HRP conjugate was added to 950 µl of assay mixture in a plastic cuvette. The rate of reaction was measured at 450 nM (Beckman SP 180) and all the results were corrected for a standard enzyme activity of 2000 units per ml per hour.

A pooled serum sample was also run with each assay to check the replication of the assay.

Coefficient of Variation

The variation between triplicates of samples were assessed by the following formula:

$$\text{Coefficient of Variation} = \frac{\text{Standard deviation}}{\text{mean}} \times 100$$

The variation between triplicate samples was less than 10%

A diagrammatic representation of the ELISA for measuring serum antibody is presented in Fig. 2.1.

2.2 Quantification of Serum Antibodies

Simple absorbtion readings from the ELISA have some validity as indicators of trends, but they are prone to systematic and random variability so it is difficult to make comparisons between parallel results. No consideration is made for the sigmoidal shape of the optical density curve.

Methods to Attempt to Quantify Serum Antibodies

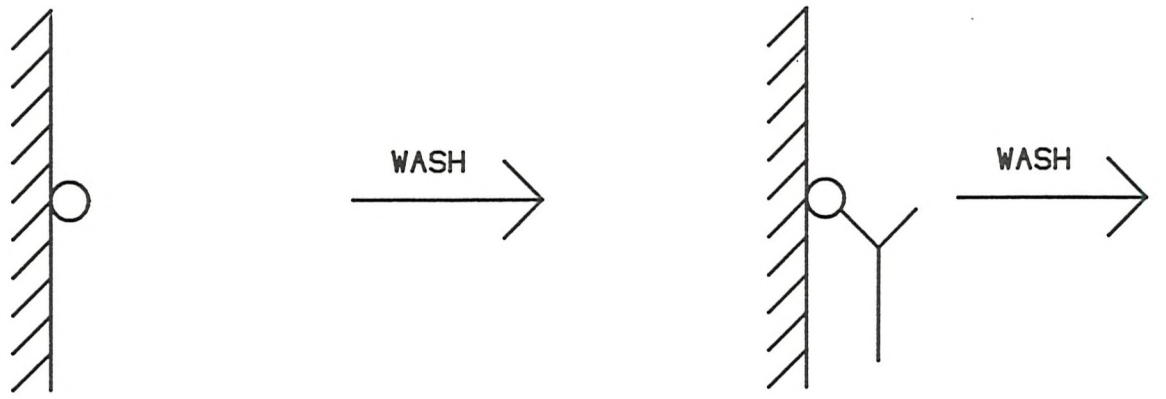
2.2.1 Purification of Specific Antibodies

Specific antibodies may be purified from rabbit antisera. A dilution curve of the purified antibodies can then be run and the optical density readings from the experimental sera can then be quantified with reference to the dilution curve.

2.2.1(i) Raising Serum Antibodies in Rabbits *(Hudson & Hay, 1980)*

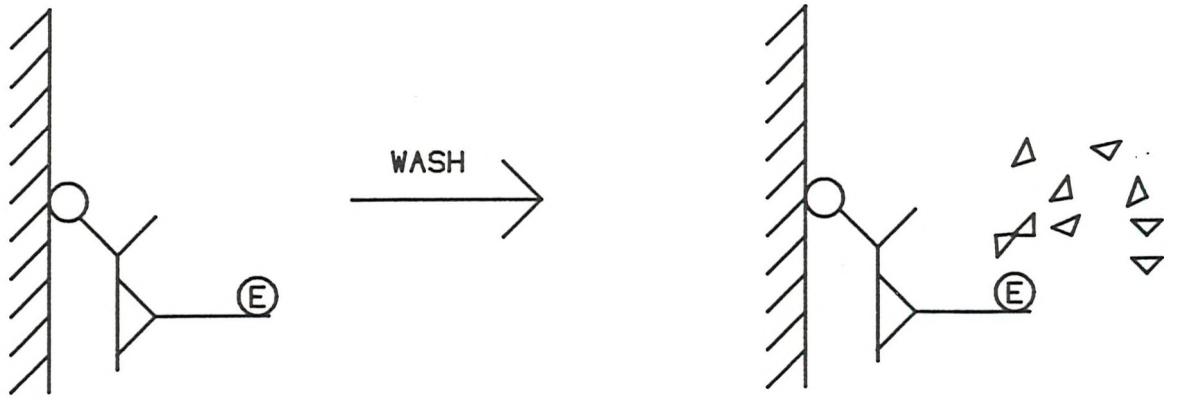
One milligram of the protein to be injected was dissolved in 1 ml of saline. An equal amount of Freunds complete adjuvant was added and an emulsion was made by forcing the mixture through a syringe needle until it was thick and white.

Female New Zealand White Rabbits (approximately weighing 2.5 kg) were used for immunization. The upper part of the hind limb was shaved and approximately 1 ml of the emulsion was injected into each side. The animals were boosted with a similar injection 4 weeks later and after a further 2 weeks a 5 ml blood sample was taken from the ear vein. The blood was allowed to clot in a glass centrifuge tube at 37^{o}C for 1 hour and then centrifuged at 3,000g for 15 minutes to collect the serum. The serum was removed by a pasteur pipette and tested for



1. ANTIGEN ADSORBED TO POLYSTYRENE SURFACE

2. INCUBATE WITH SERUM: ANY SPECIFIC ANTIBODY ATTACHES TO ANTIGEN



3. INCUBATE WITH ENZYME-LABELLED ANTI-IgG WHICH ATTACHES TO ANTIBODY

4. ENZYME ASSAY: AMOUNT OF SUBSTRATE OXIDISED = AMOUNT OF IgG PRESENT

Figure 2.1 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA). AN INDIRECT METHOD FOR ASSAY OF SPECIFIC IgG.

the presence of serum antibodies by the ELISA method given in Section 2.1.1. If antibodies were still detected when the serum was diluted 1:100,000 with PBST, the animal was anaesthetized with sagital (0.5 ml/kg) and the blood collected by cardiac puncture.

If the serum did not give a positive result at the above dilution the animal was boosted as previously, except that Freunds complete adjuvant was substituted for Freunds incomplete adjuvant. The serum was again checked for antibodies after 2 weeks.

2.2.1(ii) Isolation and Purification of Specific IgG by Immunoaffinity Chromatography

Preparation of the Immunosorbant

3g of cyanobromide activated sepherose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) was swollen and washed on a scintered glass funnel with 600 mls of HCl solution (10^{-3}M). The gel was washed in 500 mls sodium carbonate buffer (0.1 M pH 8.5 containing 0.4 M NaCl), resuspended to 50 ml and 100 mg of the specific protein was added. The protein was that which the antibodies to be purified were raised against. The solution was incubated for 16 hours at 4^{o}C with gentle shaking, then washed on a scintered glass funnel with Na HCO_3 buffer (0.1 M pH 8.5 containing 0.4 M NaCl). The immunosorbant was incubated with ethanolamine solution (1M pH 9.0) at 4^{o}C for 1-2 hours to absorb any remaining active groups. The beads were then washed in acetate buffer (0.1M pH 4.0 containing 1 M NaCl) then Na_2CO_3 buffer (0.1 M pH 8.5 containing 1M NaCl), this washing procedure was repeated three times. The immunosorbant was resuspended and stored in running buffer (PBS containing 0.02% (w/v) sodium azide) at 4^{o}C.

Purification of Specific Antibody

5 mls of the immunosorbant prepared by the method previously described was washed extensively on a scintered glass filter with PBS at room temperature. The gel was then suspended in 4 mls of PBS and 1 ml of antisera was added and gently stirred at room temperature for 1 hour. The beads were then made into a column and transferred to a cold room and kept at 4^{o}C. The column was washed with PBS until the absorbtion of the elutant at 280 nm was less than 0.02 nm.

The antibodies were then eluted with glycine-HCl buffer (0.2 M pH 2.8) and immediately neutralised in carbonate buffer (1 M pH 8.5). The eluted antibodies were dialysed against three changes of PBS containing 0.01% (w/v) sodium azide. The purified antibody was quantified by reading the optical density at 280 nm and calculated as mg protein per ml buffer.

The purification of antiovalbumin lgG antibodies was successful. However, in the case of cows milk protein, continual leakage of protein from the column occurred after the addition of the serum containing antimilk protein antibodies. This method may have been unsuccessful because of the different binding affinities of antibodies to the individual milk proteins. Also, the presence of lactose and a certain amount of fat in the milk protein which was used to bind to the beads may have prevented efficient binding. If elution of the antibodies had been obtained, it would not necessarily represent the profile of milk antibodies in the serum, again due to the different affinities of a particular milk protein antibody with antigen. Also the binding of the milk protein to the activated sepherose may not represent the antigen profile in the milk.

A different method therefore had to be used to express the optical density readings obtained from ELISA which would improve on direct absorbance readings by accounting for the sinusoidal shape of the optical density curve. The most satisfactory method was to express the results as a percentage of a standard reference serum, thus introducing a reference scale.

2.2.2 Introduction of a Reference Scale

The use of a specific antisera raised against the protein in question has been found to be the most successful method for expressing ELISA results *(Malvano, Davis & Zannino, 1982)*. The use of a reference scale was found to eliminate systematic components of error and give analytical consistency of the measurements as well as giving a more accurate comparison between results due to the consideration of the particular shape of the optical density curve.

However, the use of either of the above methods was found to be unsuitable for experiments involving mice, due to the difficulty in obtaining sufficient antiserum in mice; furthermore, antiserum could

not be obtained in humans. Therefore, the results from the experiments involving mice and humans were left as optical density readings.

2.3 An Enzyme Linked Immunosorbant Assay (ELISA) for the Detection of Serum Antigen (Fig. 2.2)

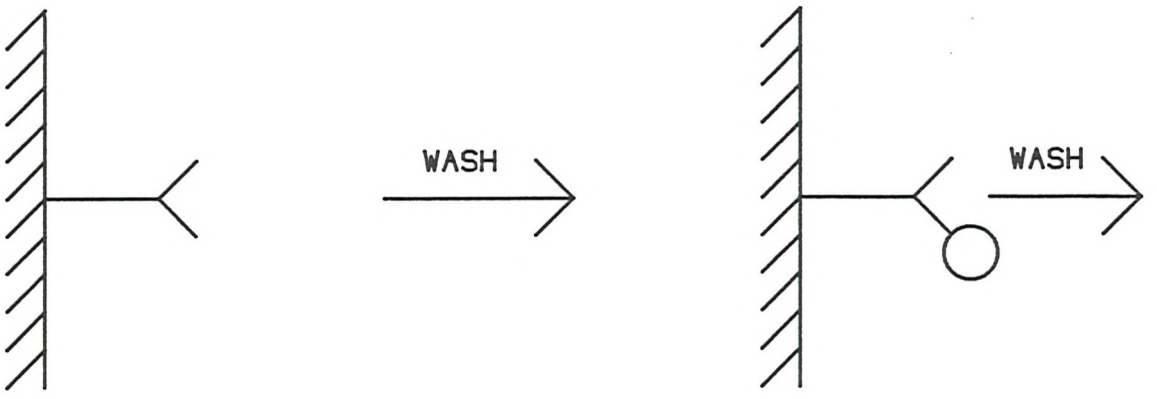
Polystyrene cuvettes were coated with a carbonate-bicarbonate buffer (50 mM pH 9.6) containing 0.02% (w/v) sodium azide and anti-specific antigen purified from antisera from immunized rabbits (2.2.1(i)).

Standard antigen samples were assayed using different coating concentrations of antispecific lgG to determine the concentration which provided the best standard curve. The cuvettes were incubated overnight in a humidity chamber at $4^{o}C$. The wells were then washed 5 times with PBST and soaked for 30 seconds between each wash (this procedure was used at each washing stage). A 200 µl aliquot of serum was added to each well, having been diluted appropriately in PBST. The serum was incubated in the wells for 2-6 hours and an antigen peroxidase conjugate was then added and incubated at room temperature for 2 hours or overnight at $4^{o}C$.

After a final washing step with PBST, the assay solution was added. The assay mixture was prepared immediately before use and consisted of a phosphate-citrate buffer (pH 5.0). The buffer contained 24.3 mls citric acid (0.1 M) and 25.7 mls disodium hydrogen phosphate (0.2M) and 50 mls of distilled water, in which was dissolved 40 mg of O-phenylene diamine and 40 µl of a 30% (v/v) H_2O_2 solution was added. The colour was left to develop for exactly 15 minutes, then stopped by the addition of 200 µl of citrate-phosphate buffer (0.3 M pH 2.85).

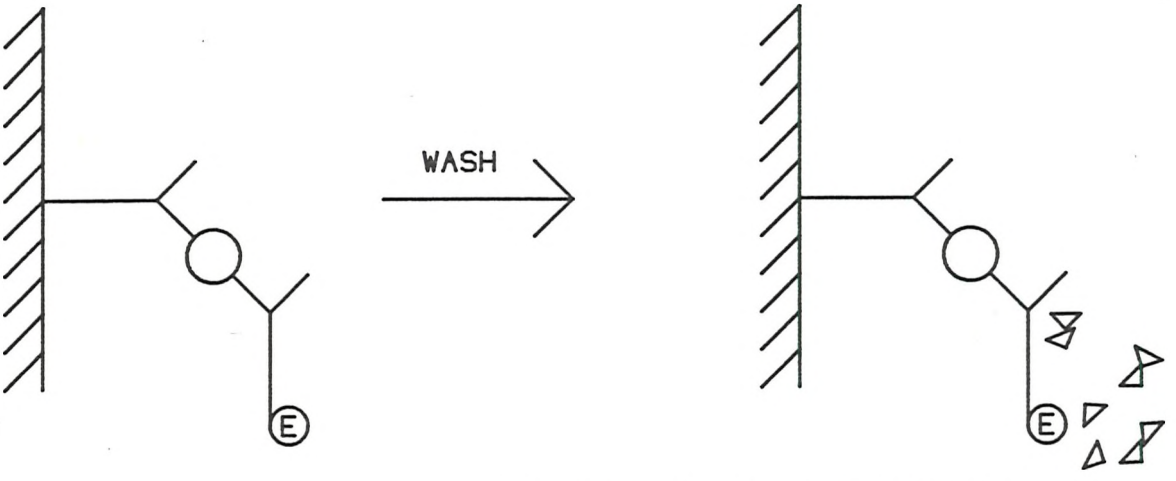
The optical density of the contents was read at 405 nm by the ElA Manual Reader. The concentration of antigen in the serum was calculated from a standard curve consisting of the same antigen.

The standard curve consisted of a range of antigen concentrations (100 µg to 1.0ng per ml) diluted in PBST and normal rabbit serum which did not contain any of the specific antigen.



1. ANTIBODY ADSORBED TO POLYSTYRENE SURFACE.

2. INCUBATE WITH SERUM CONTAINING ANTIGEN: ANTIGEN ATTACHES TO SPECIFIC ANTIBODY



3. INCUBATE WITH ENZYME LABELLED SPECIFIC ANTIBODY

4. ENZYME ASSAY: AMOUNT OF SUBSTRATE OXIDISED = AMOUNT OF ANTIGEN PRESENT.

Figure 2.2 DOUBLE ANTIBODY SANDWICH ELISA FOR MEASURING ANTIGEN.

2.3.1 Purification of Serum IgG for the Antigen Assay

A solution of 3% Rivanol (6,9 Diamino-2-ethoxy acridine lactate, Sigma Chemical Company, St. Louis, MO, USA) (w/v) in distilled water was prepared and added to the serum at a volume of 65% of the volume of serum. The serum was obtained from rabbits that had been immunized with the specific antigen which was then diluted in distilled water (69% volume of distilled water added to 1 volume of serum).

The solution was left for 15 minutes at room temperature and then spun at 3000g for 15 minutes. The resulting precipitate was discarded and 5% (w/v) NaCl was added to the supernatant, which was then stirred and left at room temperature for 15 minutes. The precipitate was removed by filtration on fluted filter paper and to the filtrate an equal volume of saturated ammonium sulphate solution was added. After 30 minutes the solution was spun at 3000g for 20 minutes and the supernatant discarded. The resulting precipitate was washed in 5 mls of a 50% saturated ammonium sulphate solution and spun again at 3000g for 20 minutes. A further 5 mls of 50% saturated ammonium sulphate solution was added and this was dialysed extensively in carbonate buffer (0.01 M pH 8.0) at $4^{o}C$.

The purified IgG in carbonate buffer was stored in 0.5 ml aliquots at $-22^{o}C$.

2.3.2 Conjugation of Anti-Antigen IgG and Horse Radish Peroxidase

Conjugates were prepared by the following method for the antigen assay. Rabbits were immunized as described in Section 2.2.1(i) and the serum IgG was purified by the method described in Section 2.3.1.

2.3.2(i) A Periodate Method of Conjugation was used which was based on the procedure developed by *Nakane and Kawoi (1974)*.

Sigma HRP Type VI (5 mg) was dissolved in 1 ml of sodium bicarbonate buffer (0.3 M pH 8.1). A solution of 1 fluoro 2,4 dinitrobenzene (Koch Light Laboratories, Coinbrook, Buckinghamshire) in ethanol was added and the mixture allowed to stand at room temperature with mixing. After 1 hour 1 ml of freshly made $NaIO_4$ solution (0.08 M in distilled water) was added and the mixture allowed to stand at room temperature for 30 minutes during which time a yellow-

green colour developed. 1 ml of 1% (v/v) ethylene glycol in distilled water was added and the mixture allowed to stand at room temperature for 1 hour. The solution was dialysed 3 times against 1 litre of sodium carbonate buffer (0.01 M, pH 9.5) at 4°C. To the peroxidase aldehyde formed 5 mg IgG in 1 ml carbonate buffer (0.01 M, pH 8.0) was added and the mixture was allowed to stand at room temperature for 3 hours. To stabilize the Schiff bases 2 mg per ml $NaBH_4$ (Sigma Chemicals) was added and the mixture allowed to stand again for 3 hours at 4°C. To this an equal amount of saturated ammonium sulphate was added and the mixture stirred for 15 minutes at room temperature, then left overnight at 4°C to allow the precipitate to develop. The precipitate was washed twice with 50% saturated ammonium sulphate solution and re-dissolved in 1 ml water and extensively dialysed against PBS at 4°C.

An equal volume of glycerol was added to the conjugate and stored at -22°C.

2.4 Clq Binding Test for the Detection of Immune Complexes in Serum

This method was used to measure circulating immune complexes as it has been demonstrated to be efficient at measuring complexes of the intermediate size *(Jones et al., 1982)*.

The procedure used was based on the method of *Zubler et al. (1976)*. The assay relies on the ability of soluble immune complexes to fix a subunit of the first component of the complement cascade. Immune complexes bind to the first component of the complement cascade Clq via the Fc portion of the antibody in the immune complex.

The bound complex containing Clq can be precipitated in a solution of 3% polyethylene glycol. To quantify the presence of immune complex the Clq was radiolabelled.

2.4.1 Isolation and Purification of Clq *(Volankis & Stroud, 1972)*

The isolation and purification of Clq involved a series of precipitation steps in ethylene diamine tetracetic acid (EDTA) (EDTA -disodium salt, BDH Ltd., Poole, Dorset). 180 mls of freshly drawn blood was allowed to stand for 1 hour at room temperature then 1 hour at 4°C. The blood was spun at 1500 g for 15 minutes to separate the serum. The serum was spun at 30,000g for 30 minutes to

remove the lipid. 40 mls of serum was incubated with 10 mls EDTA (0.1 M, pH 7.5) for 10 minutes at 37°C. The solution was kept cool in an ice bucket and 200 mls of EDTA (0.005 M, pH 7.5) was added slowly and incubated at 4°C for 1 hour and stirred every 20 minutes for 1 minute. The free Clq was collected by centrifuging at 12,000g for 30 minutes in 8 x 35 ml centrifuge tubes. The supernatant was discarded and a white precipitate was visible on the side walls of the centrifuge tubes. To the tubes, 5 mls EDTA (0.022 M, pH 7.5) was added and the precipitate was scraped thoroughly off the side walls with a small spatula. The fluid containing the precipitate was pooled into 2 x 35 ml centrifuge tubes and again spun at 12,000g for 30 minutes. The resulting supernatant was again discarded, and the precipitate was again scraped into 5 mls of EDTA (0.022 M, pH 7.5) and combined into one 3 5 ml centrifuge tube. The precipitate was spun at 12,000g for 30 minutes, the supernatant discarded and the precipitate was dissolved in 10 mls EDTA (0.01 M, pH 5.0 containing 0.75 M NaCl) and left overnight at 4°C.

The solution was spun at 30,000g for 30 minutes and the supernatant was dialysed for 4 hours in 1 litre of EDTA (0.1 M, pH 5.0), the buffer was changed and fresh buffer added after 2 hours.

The contents of the dialysis bag were centrifuged at 12,000g and the precipitate was resuspended in 15 mls EDTA (0.1 M, pH 5.0). This cycle was repeated twice combining the precipitate into 1 x 15 ml centrifuge tube. The precipitate was finally dissolved in 1 ml EDTA (0.1 M, pH 7.5 containing 0.3 M NaCl) and dialysed extensively against veronal buffered saline (VBS) (pH 7.5) and concentrated by ultra filtration to 0.5 mg protein per ml in VBS and stored at -90°C.

2.4.2 Radioiodination of Clq

The method employed was a modification of the procedure developed by *Salacinksi et al (1979)*.

A glass scintillation vial was coated with 50 µl of iodogen (1,3,4,6-tetrachloro-3,6,-diphenylglycouril, Pierce Chemical Company, Rockford, USA) dissolved in chloroform (1 mg 1 ml). To the vial a 200 µl aliquot of Clq (100 µg) in VBS was added with 10 µl (1mCi) of sodium-^{125}Iodine (The Radiochemical Centre, Amersham, Buckinghamshire). The vial was incubated at room temperature for 10 minutes. The solution

was then passed down a column packed with sephedex G25 washing with VBS. The first radioactive peak was collected in 0.5 ml aliquots and 0.5% ovalbumin was added as a protein carrier. The radiolabelled Clq was stored in lead at -70°C.

2.4.3 Clq Binding Assay (Illustrated in Fig. 2.3)

A 50 µl aliquot of ^{125}I Clq solution was added to 4.95 ml VBS containing 0.5% ovalbumin. The solution was centrifuged at 12,000g for 20 minutes to remove complexed Clq. The top 4 mls of supernatant was removed for the assay.

Triplicate 50 µl samples of the experimental serum was aliquoted into plastic tubes (LP_3 - Luckham Ltd., Burgess Hill, Sussex) together with 100 µl of EDTA (0.2 M, pH 7.3). The tubes were incubated at 37°C for 30 minutes to decomplement the serum and then placed on ice.

A 50 µl aliquot of ^{125}I Clq in VBS was added to each tube followed immediately by the addition of 1 ml 3% polyethylene glycol (PEG 6000, Sigma Chemicals Ltd., Poole, Dorset) in borate buffer (0.1 M, pH 8.3). Each tube was capped and vortexed for 5 seconds then incubated on ice with gentle shaking for 1 hour.

Separation of unbound Clq was achieved by centrifuging at 25,000g for 20 minutes at 4°C. The supernatant was removed and the pellet was washed in a further 1 ml of 3% PEG in borate buffer. After recentrifuging at 25,000g for 20 minutes the supernatant was again removed by a suction pipette and the radioactivity present in the precipitate was determined by gamma counting for 1 minute and the results were expressed as a percentage of the counts resulting from precipitation of 50 µl of ^{125}I labelled Clq by 20% Trichloroacetic acid.

Standardization

The assay was standardized by running a series of concentrations of alkali-aggregated human and globulin with each test.

Triplicate 50 µl samples of pooled normal human serum containing 50 µl of aggregated human gamma globulin diluted to various concentrations in PBS were incubated with 50 µl EDTA (0.2 M, pH 7.3) at 37°C for 30 minutes along with experimental serum. The control aggregated human gamma globulin containing tubes continued to be treated along with the experimental sera.

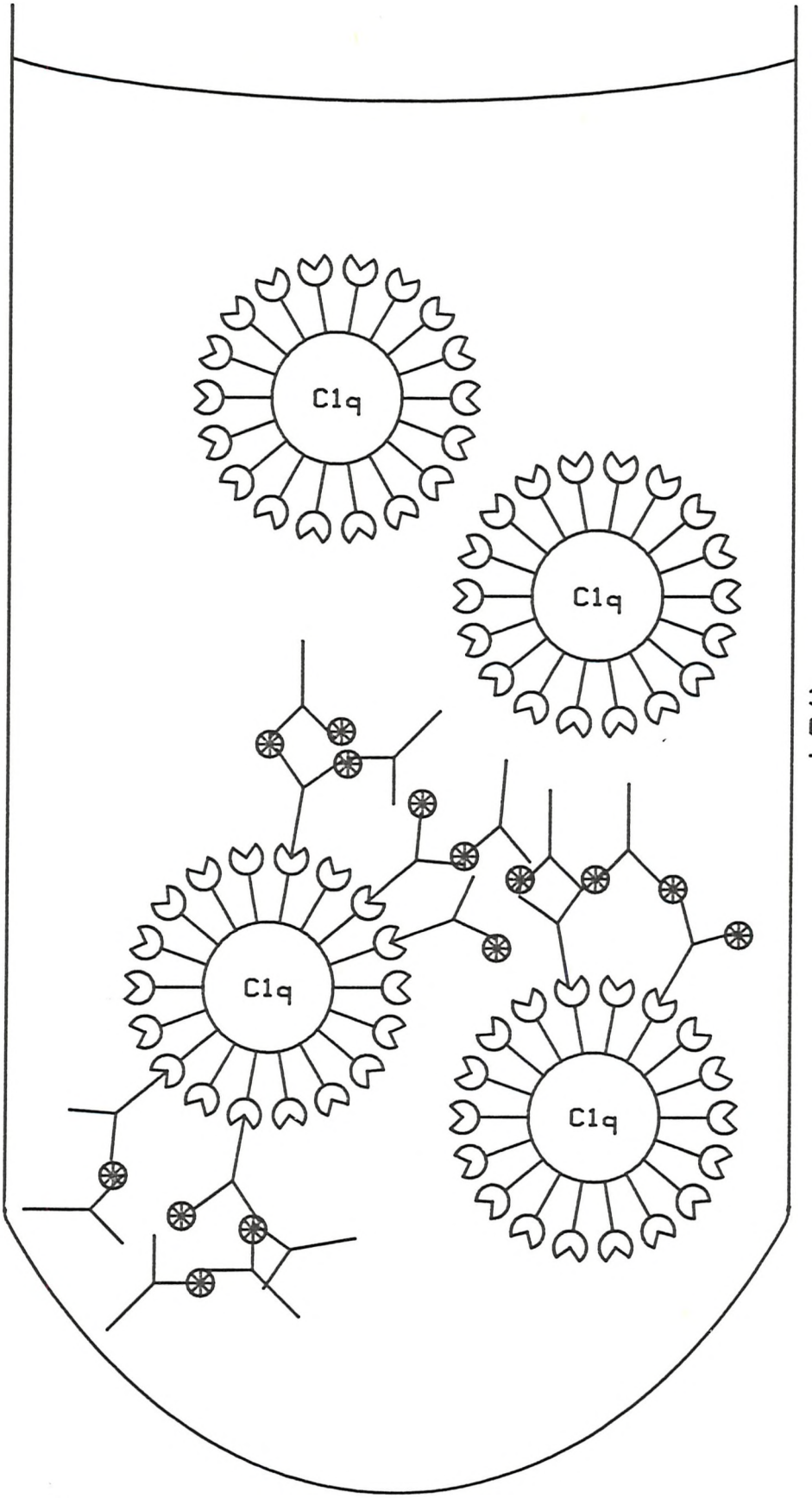


Figure 2.3 SUMMARY OF THE REACTIONS INVOLVED IN THE C1q BINDING RADIOIMMUNE ASSAY.
- PURIFIED C1q COMBINES WITH IMMUNE COMPLEXES PRESENT IN THE SERUM SAMPLE TO FORM A COMPLEX WHICH IS INSOLUBLE IN 3% PEG.

2.4.4 Preparation of Human or Rabbit IgG Aggregates

Rabbit or human IgG Cohn Fraction II (Sigma Chemicals, Poole, Dorset) at a concentration of 20 mg/ml was mixed with an equal volume of sodium hydroxide (0.2 M) in an ice bath and constantly stirred for 1 hour.

The solution was then dialysed against several changes of PBS to give a final pH of 7.4.

The protein concentration was determined by the Lowry method *(Lowry, 1951)* and aliquots of aggregates were stored at -70°C.

2.5 Automated Procedure for Cholesterol and Triglyceride Determination

Samples for the autoanalyser (Technicon Instruments Corp. Ltd., Tarrytown, N.Y., 1972) were prepared by pipetting 100 µl of serum into 1.9 ml of 99% isopropranolol and 2.0g of zeolite (Sigma Chemicals, St. Louis, Miss.) in disposable 2.5 ml plastic tubes. The tubes were stoppered vortexed and spun at 1000g for 10 minutes. The supernatant was decanted into stoppered polyethylene vials for cholesterol and triglyceride analysis by the automated procedure.

(Technician Clinical Methods, No. 16A, September 1972, Nos. 24, March 1972).

2.6 Animals

Rabbits

New Zealand White Rabbits both male and female were weaned from the colony at Southampton University which were reared on a commercial stock diet (Labsure Animal Foods, Christopher Hill Group Ltd., P.O. Box 6, Castle Street, Poole, Dorset).

The animals were weaned at 6 - 8 weeks post partum and randomly allocated to different experimental groups. All the rabbits were housed individually in stainless steel cages and allowed free access fo food and water, all of the experimental diets were fed ad libertum.

Mice

Male and female mice were from the University of Southampton colony which had been reared on a commercial chow diet (PRD - Christopher Hill Group, Dorset, U.K.)

The mice were weaned at 21 days and housed in groups of up to 10 mice per cage. The mice were allowed free access to food and water at all times.

All animals were maintained in rooms in which the temperature was kept at 21°C and the lighting followed a 12 hour light and dark cycle.

2.6.1 Collection of Blood Samples

Blood samples were taken from the marginal ear vein in the rabbit. The ear was shaved with a scalpel blade to expose a portion of the vein in which a small cut was made by the scalpel blade. The vein was pinched below the cut and the blood was allowed to drip freely into a glass centrifuge tube. Approximately 4 mls of blood was collected at each bleed and at the end of the bleed a piece of gauze was placed over the nicked vein and pressure was applied until the bleeding stopped.

Blood samples from the mice were obtained by decapitation. After decapitation the mouse was quickly placed in a funnel and the blood was allowed to drip into an LP_3 tube (Luckham Ltd., Burgess Hill, Sussex). The blood from three mice was pooled to make one sample.

2.7 Experimental Diets

Preparation of Diets

Rabbit Diets The experimental diets for rabbits were prepared by mixing the dry ingredients in a Hobart mixer and pelleted after the addition of water (10% (v/w)). The resulting mixture was then pelleted to a size of 3 x 20 mm and dried overnight at 60°C in a forced draught oven.

When the diet involved the addition of a particular ingredient to the normal rabbit stock diet (RAG stock diet, Labsure Animal Foods) the stock pellets were ground up to a fine powder, the additional ingredient such as dried skimmed milk or powdered ovalbumin was added with 10% water, repelleted and dried as previously described.

Mice Diets The experimental diets for mice were prepared by mixing the dry ingredients in a Hobart mixer and after thorough mixing, water was added until the ingredients formed a soft but not sticky dough.

The dough was then passed through a mixer attached to the Hobart mixer forming pellets of 10 x 30 mm which were dried on trays overnight at 40°C in a forced draught oven.

2.8 Statistical Methods

The data were analysed by either one-way or two-way analysis of variance (ANOVA). Mean values are presented in the tables accompanied by their appropriate standard error. However, comparison of means was carried out using pooled standard error of the means, following ANOVA by either Duncans Multiple Range Test or the Least Significant Difference Test as outlined by *Snedecor (Statistical Methods, 7th Edition, Iowa State Univ. Press)*.

The data for rabbit antigen specific antibodies is expressed as a percentage of the appropriate antiserum (See Section 2.2.2). This required angular transformation prior to ANOVA, since the statistical analysis for data based on percentages is inadvisable.

TABLE 2.1

Rabbit Stock Diet RAG*

		Content g/kg Diet
Oats		125
Wheat Feed		134
Bran		375
Linseed		50
Fish Meal (English White Fish)		100
Dried Grass		200
Embazin Premix		1.12
RAG Vitamins	(See Table 2.1.1)	5.0
RAG Minerals	(See Table 2.1.2)	10

Metabolizable Energy = 7.85 MJ/kg

Crude Protein = 208.2 g/kg Diet

*Labsure Animal Foods, Christopher Hill Group Ltd., P.O. Box 6, Castle Street, Poole, Dorset

TABLE 2.1.1

Contents of RAG Vitamin Mix (1 Kg)

Vitamin A	-	1600000 iu
Vitamin D_3	-	200000 iu
Vitamin B_2	-	1.6 g
Nicotinic Acid	-	4 g
Pantothenic Acid	-	0.8 g
Vitamin B_{12}	-	2.4 mg
Vitamin E	-	5000 iu
Vitamin K	-	2 g
Folic Acid	-	1.2 g
Choline Chloride	-	40 g
Vitamin B_1	-	0.4 g

Made up to 1 Kg by the addition of Fine Maize Meal

TABLE 2.1.2

RAG Mineral Mix

	g/Kg Mineral Mix
Sodium Chloride	201
Limestone Flour	558
Dicalcium Phosphate	156.4
Magnesium Oxide	16.7
Iron Sulphate	14.7
Copper Sulphate	2.7
Manganese Sulphate	35.5
Cobalt Sulphate	0.19
Calcium Iodate	0.23
Fine Maize Meal	14.5

TABLE 2.2

Stock Diet + 10% Dried Skimmed Milk

100g Dried Skimmed Milk (St. Ivel Ltd., Trowbridge, Wilts) per kilogram of RAG Stock Diet (Labsure Animal Foods, Christopher Hill Group Ltd., P.O. Box 6, Castle Street, Poole, Dorset).

Milk Protein per Kg Diet

Total Milk Protein	36.4 grams
Casein	31.01 grams
β Lactoglobulin	3.69 grams
α Lactalbumin	1.31 grams
Bovine Serum Albumin	0.109 grams

TABLE 2.3

Stock Diet + Casein

RAG Stock Diet (Labsure Animal Foods, Christopher Hill Group Ltd., P.O. Box 6, Poole, Dorset)

Casein (Express Dairy Foods Ltd., 430 Victoria Road, South Ruislip, Middlesex, HA4 OHF)

a) RAG Stock Diet + 1% Casein (10 g Casein per kg stock diet)
b) RAG Stock Diet + 5% Casein (50 g Casein per kg stock diet)
c) RAG Stock Diet +10% Casein (100g Casein per kg stock diet)

TABLE 2.4

Stock Diet + Hen Ovalbumin

RAG Stock Diet (Labsure Animal Foods, Christopher Hill Group Ltd., P.O. Box 6, Poole, Dorset)

HEN Ovalbumin (John F. Renshaw & Co., Ltd., Mitcham, Surrey, England)

a) RAG Stock Diet + 1% Ovalbumin (10 g Ovalbumin per kg stock diet)

b) RAG Stock Diet + 5% Ovalbumin (50 g Ovalbumin per kg stock diet)

c) RAG Stock Diet + 10% Ovalbumin (100 g Ovalbumin per kg stock diet)

TABLE 2.5

Semi Purified Cows Milk Rabbit Diet

	g/Kg Diet
Casein	140 (Express Dairy Foods Ltd)
Dried Skimmed Milk	506 (St. Ivel Ltd., Trowbridge)
Bran	150
Coconut Oil	158
Maize Oil	21
Methionine	1
Minerals (See Table 2.5.1)	23
Vitamins (See Table 2.5.2)	9

Metabolisable Energy	=	18 MJ/Kg
Energy From Protein	=	31%
Energy From Fat	=	40%
Energy From Cabohydrate	=	29%
Total Cholesterol Content	=	0.005%

TABLE 2.5.1

Minerals

		g/Kg Diet
$CaHPO_4$ DCP	=	16.4
$CaCO_3$	=	4.6
$Mg(OH)_2$	=	1.22
$CuSO_4$	=	0.015
$FeSO_4$	=	0.678
$MnSO_4$	=	0.38
$ZnSO_4$	=	0.25
KlO_3	=	0.00813
$NaMoO_4$	=	0.0043
$CrKO_4$ $12H_2O$	=	0.0092
Na_2Sa $1OH_2O$	=	0.0045

TABLE 2.5.2

Vitamins

	mg/Kg Diet
Biotin	0.097
Folic Acid	2.36
Inositol	1.74
Nicotinic Acid	47.44
Ca Pantothenic Acid	9.26
Vitamin A	10.17
Vitamin D	4.08
Rovermix E_{25}	315.14
Menapthone K	3.58
Thiamine	1.75
Maize Starch	7.77 g
Choline	1.13 g

TABLE 2.6

Laboratory Mouse Stock Diet

(PRD - From Christopher Hill Group, Dorset, U.K.)

Diet Composition (g/Kg)

Ground Barley	51.25
Ground Wheat	200.00
Ground Maize	100.00
Ground Oats	181.25
Wheat Feed	200.00
Soya Extracts	100.00
Dried Skimmed Milk	75.00
Dried Yeast	25.00
White Fish Meal	50.00
Sodium Chloride	7.50
Vitamin Supplement	5.00
Mineral Mixture	500
Energy From Protein	19.7%

TABLE 2.7

Mouse Diet 1 (AIN 1976)

	g/Kg
Ovalbumin	203
Corn Starch	150
Sucrose	500
Bran	50
Corn Oil	50
AIN Minerals (Table 2.7.1)	35
AIN Vitamins (Table 2.7.2)	12

Energy from Protein	=	21%
Energy from Carbohydrate	=	67%
Energy from Fat	=	11%

TABLE 2.7.1

Mineral Mix (AINS, 1976)

	g/Kg
$CaHPO_4$	500
NaCl	74
KCl	42
K_2SO_4	52
$Mg(OH)_2$	35
Manganous Carbonate	3.5
Ferric Citrate	6.0
Zinc Carbonate	1.6
Cupric Carbonate	0.3
Kl	0.01
NaSe	0.01
$Cr(K)(SO_4)_2$	0.55
$NaMO_4$	0.01
Sugar	285

TABLE 2.7.2

Vitamin Mix (AINS, 1976)

	Mg/Kg
Thiamine	600
Riboflavin	600
Vitamin B_6	700
Nicotinic Acid	3000
Ca Pentothenate	1600
Biotin	20
Folic Acid	200
Vitamin B_{12}	1
Vitamin A (400,000 iu)	800
Vitamin D (10,000 iu)	20
Vitamin E (5,000 iu)	20,000
Vitamin K (Menapthone)	5
Inositol	10,000
Choline	10,000
Starch Supplement	952.454 g

TABLE 2.8

Mouse Diet 2

	2.8b Experimental g/Kg	2.8a Control g/Kg
Ovalbumin	100	-
Promine D	96.6	196.6
Corn Oil	20.2	20.2
Sugar	100	100
Starch	588.4	588.4
Bran	50	50
AIN Vitamins (Table 2.7.1)	12	12
AIN Minerals (Table 2.7.2)	35	35
Energy from Ovalbumin	10.7%	0%
Energy from Total Protein	21%	21%
Energy from Carbohydrate	74%	74%
Energy from Fat	5%	5%

(The Hen Ovalbumin for the Mouse Diet 1 and 2 was purchased from John F. Renshaw & Co. Ltd., Mitcham, Surrey).

CHAPTER 3

THE IMMUNOLOGICAL RESPONSE TO DIETARY ANTIGEN - THE EFFECT OF AGE AT ANTIGEN INTRODUCTION

Introduction

Intestinal absorbtion of macromolecules is believed to be greater in the newborn than in the adult, which is probably due to the immaturity of the neonatal mucosal barrier *(Walker, 1978)*.

Most macromolecular transport studies have investigated the mechanism by which immunoglobulins are transported to the neonate using animals such as the rat, which receives the majority of its passive immunity post-natally, or the ungulate which receives all its maternal antibodies after birth.

In contrast to the transport of maternal antibodies in these species, few studies have investigated macromolecular transport in animals which receive complete interuterine passive immunity such as the rabbit and primate.

The observation in humans that infants have higher serum antibody titres particularly to cows milk suggests a period of increased macromolecular absorbtion. Peak serum antibody titres to milk proteins have been found between 3 and 12 months after birth *(Kletter et al., 1971)*, which may coincide in some infants with a transient secretory lgA deficiency *(Taylor et al., 1973; Soothill et al., 1976)*. The factors responsible for the maturation of the lgA system are not known although lgA and lgG synthesis appears to be dependent upon antigenic stimulation *(Vitella et al., 1974)*. lgA is usually the last immunoglobulin to appear after birth *(Martin & Leslie, 1973)* and the lgA system in the intestinal tract is not fully developed before 2 years of age *(Savilahti, 1972)*. Therefore, the immune response produced to a dietary protein at weaning may reflect the immaturity of the intestinal mucosa or the lack of a fully developed secretory antibody system which prior to weaning would have been supplied via the maternal colostrum and milk.

A recent study in rabbits shows a decline in macromolecular absorbtion of the protein BSA with increasing age *(Udall & Pang et al., 1981)*, indicating that the immune response to a dietary protein introduced to the more mature animal may differ from that exhibited by the weanling.

Experiment 3a investigates the production of circulating antibodies in rabbits introduced to cows milk at different stages of maturity. This is extended in experiment 3b which examines the effect of age of antigen introduction on maternally induced hyporesponsiveness.

Experiment 3a

Experimental Design

Fifteen New Zealand White Rabbits were at weaning divided into three groups, consisting of five animals; weaning was at 6 weeks post-partum. Each group of five rabbits was introduced to a diet containing milk protein at different ages.

The rabbits were fed a stock diet (Table 2.1) until they reached the designated age for the introduction of milk protein to the diet. The diet containing milk protein was made by adding 10% (w/w) of dried skimmed milk (DSM) to the stock diet (Table 2.2).

Group 1 - Introduced to DSM at weaning
(6 weeks post partum)

Group 2 - Introduced to DSM at 6 weeks post weaning

Group 3 - Introduced to DSM at 12 weeks post weaning

The rabbits were housed as outlined in Section 2.6 and had ad limitum access to fresh food and water. A blood sample was taken from the marginal ear vein at weaning and every three weeks thereafter. The blood was allowed to stand for one hour at 37°C and centrifuged at 1500g for 20 minutes. The serum was removed by a pasteur pipette and stored in 0.5 ml aliquots at -22°C, ready for analysis. Body weights were also recorded at regular intervals. Circulating serum antimilk protein lgG was measured by the ELISA Method (i), as explained in Section 2.1.1, for which the serum was diluted in PBST 1:200. The antigen used to coat the cuvettes was DSM (St. Ivel Ltd., Trowbridge) at a concentration of 30 µg per ml of coating buffer, and swine anti rabbit lgG (Nordic Immunochemical Laboratories, Tilberg, The Netherlands) was used as the enzyme label with O-phenylene diamine as the chromogen.

The optical density results obtained for the presence of circulating anti milk protein antibodies in the serum samples were expressed as a percentage of a reference serum containing antibodies raised against DSM (Section 2.2.1(i)) and measured by the same ELISA system. The standard curve for the reference serum is given in Fig. 3.1.

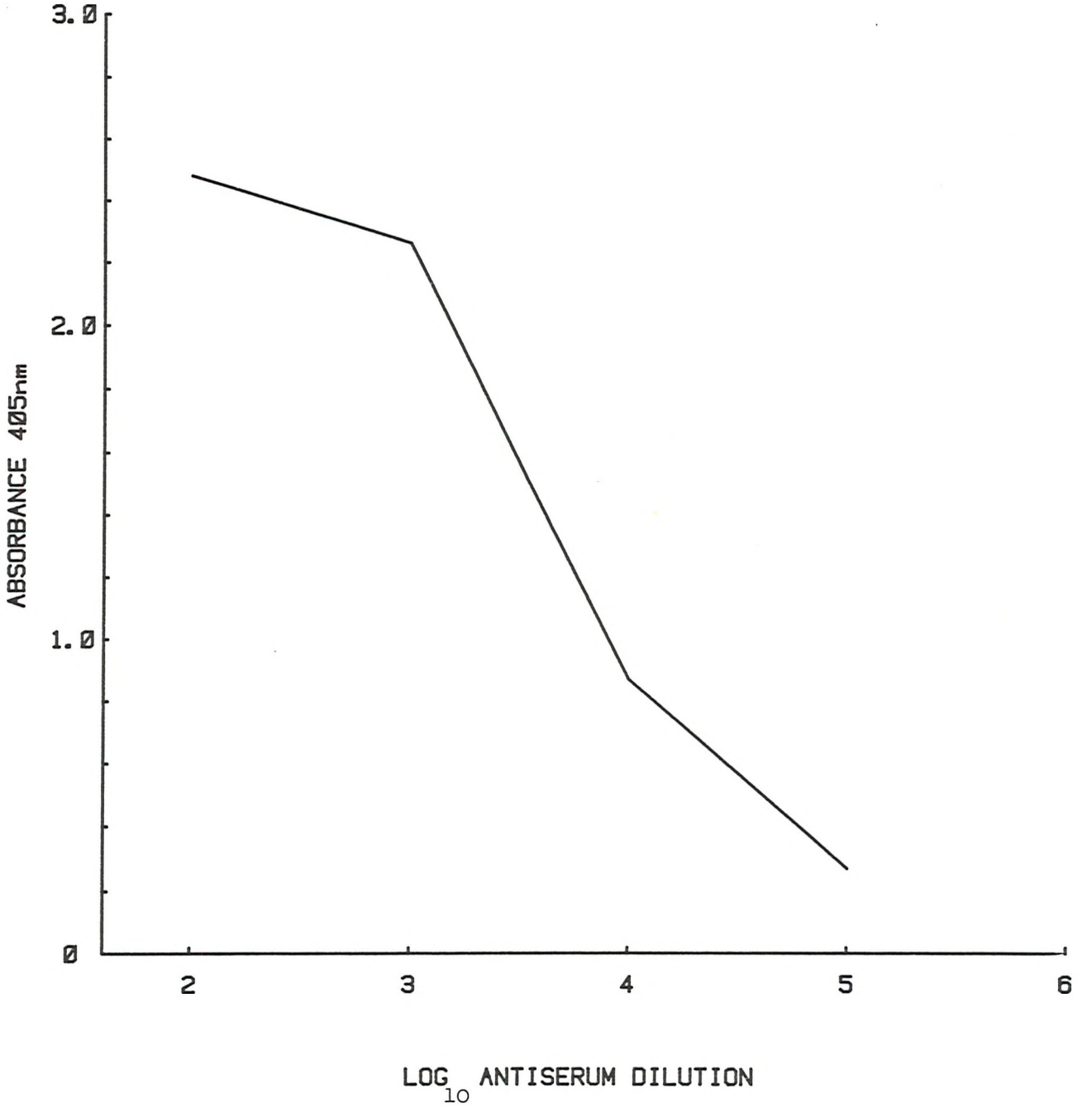


Figure 3.1 STANDARD CURVE. ANTI-DSM IgG MEASURED BY ELISA IN SERUM FROM AN ANIMAL IMMUNIZED AGAINST DSM.

Results

There was no significant difference in the body weights of the animals in each group at weaning (1.18 kg ± 0.047; 1.18 kg ± 0.024; 1.09 kg ± 0.052). The mean body weights for each group over the experimental period are given in Table 3.1.

Circulating serum anti milk lgG antibodies were analysed by two-way analysis of variance which showed that, while there was no overall difference between the groups, there was however a significant increase in antibodies during the experiment ($P < 0.001$) and that a significant group-time interaction occurred ($P < 0.01$). When the age of introduction to the milk was taken as the baseline result and the increase in specific serum antibodies over the subsequent 6 weeks examined by a two-way analysis of variance, no significant difference was found between groups, nor was the group-time interaction significant. The increase in antibodies over the experimental period remained statistically significant ($P < 0.001$).

The results within each group were further examined by the Duncan Multiple Range Test which is summarized in Table 3.2. Only the rabbits introduced to milk at 6 or 12 weeks past weaning produced a significant increase in circulating antimilk antibodies during the experimental period. Group 1, in which the milk protein was introduced to the rabbits at weaning, did not produce a significant rise in serum antibodies over the complete experimental period (Fig. 3.2).

The Duncan Multiple Range Test was also used to compare the means when the results obtained at the age of introduction of milk protein were taken as the baseline measurement of circulating antibodies. This is given in Table 3.3. Group 3, in which the rabbits were introduced to DSM 12 weeks post weaning was the only group that showed a significant increase in antibodies over the following 6 weeks. However, the result for Group 2 after a 6 week period of consuming the milk diet was not available. The mean concentration of circulating serum antibodies to milk protein of Group 3 after 6 weeks on the diet was significantly greater than for Group 1.

TABLE 3.1

Mean Body Weights (given in kgs ± S.E.) of the Rabbits in Each Group over the Experimental Period

Group	Age (Post Weaning) 0	3	6	9	12	15	18
1	1.18 ± 0.047	1.80 ± 0.048	2.41 ± 0.063	2.81 ± 0.076	2.90 ± 0.187	3.27 ± 0.098	3.41 ± 0.091
2	1.18 ± 0.024	1.81 ± 0.052	2.36 ± 0.044	2.86 ± 0.067	3.18 ± 0.038	3.40 ± 0.048	3.51 ± 0.027
3	1.09 ± 0.052	1.74 ± 0.061	2.30 ± 0.075	2.70 ± 0.11	3.02 ± 0.114	3.30 ± 0.136	3.38 ± 0.140

No significant difference was found in the mean weight gain of each group of animals

TABLE 3.2

Mean Values (± S.E.) for Circulating IgG Antibodies to Dietary Cows Milk Protein in Rabbits Introduced to Cows Milk Protein at Weaning (Group 1) or at 6 weeks (Group 2) or 12 weeks (Group 3) Post Weaning

Age (Weeks Post Weaning)	Group 1	Group 2	Group 3
0	0.428 ± 0.208[a]	0 ± 0[a]	0.360 ± 0.134[a]
3	2.99 ± 1.84[a]	0.072 ± 0.072[a]	0.504 ± 0.211[a]
6	1.622 ± 0.605[a]	0.396 ± 0.170[ab]	0.660 ± 0.180[a]
9	1.616 ± 0.636[a]	5.106 ± 3.73[ab]	0.319 ± 0.095[a]
12	0.824 ± 0.186[a]	N.A.	0.728 ± 0.066[a]
15	1.068 ± 0.263[a]	3.83 ± 2.69[ab]	2.622 ± 0.812[a]
18	0.918 ± 0.314[a]	11.90 ± 9.57[b]	29.96 ± 17.55[b]

The results are expressed as a percentage of a standard reference serum (Fig. 3.1)

a,b - Mean values in any group without a common superscript differ significantly ($P < 0.05$)

NA - Not available

TABLE 3.3

Mean Values (± S.E.) for Circulating IgG Antibodies to Dietary Cows Milk Protein in Rabbits Introduced to Cows Milk Protein at Weaning (Group 1) or at 6 Weeks (Group 2) or 12 Weeks (Group 3) Post Weaning

Time (Weeks) After Introduction of Cows Milk Protein	Group 1	Group 2	Group 3
0	0.428 ± 0.208[a]	0.396 ± 0.170[a]	0.728 ± 0.066[a]
3	2.99 ± 1.84[a]	5.106 ± 3.73[a]	2.622 ± 0.872[a]
6	1.616 ± 0.636[a]	N.A.	29.96 ± 17.55[b*]

The results are expressed as a percentage of a standard reference serum (Fig. 3.1), and compare values at introduction of cows milk protein and 3 and 6 weeks post-introduction.

a,b - Mean values in any given group without a common superscript differ significantly ($P < 0.05$)

* - Differs significantly from any other group mean value for a given time after antigen introduction ($P < 0.05$)

NA - Not available

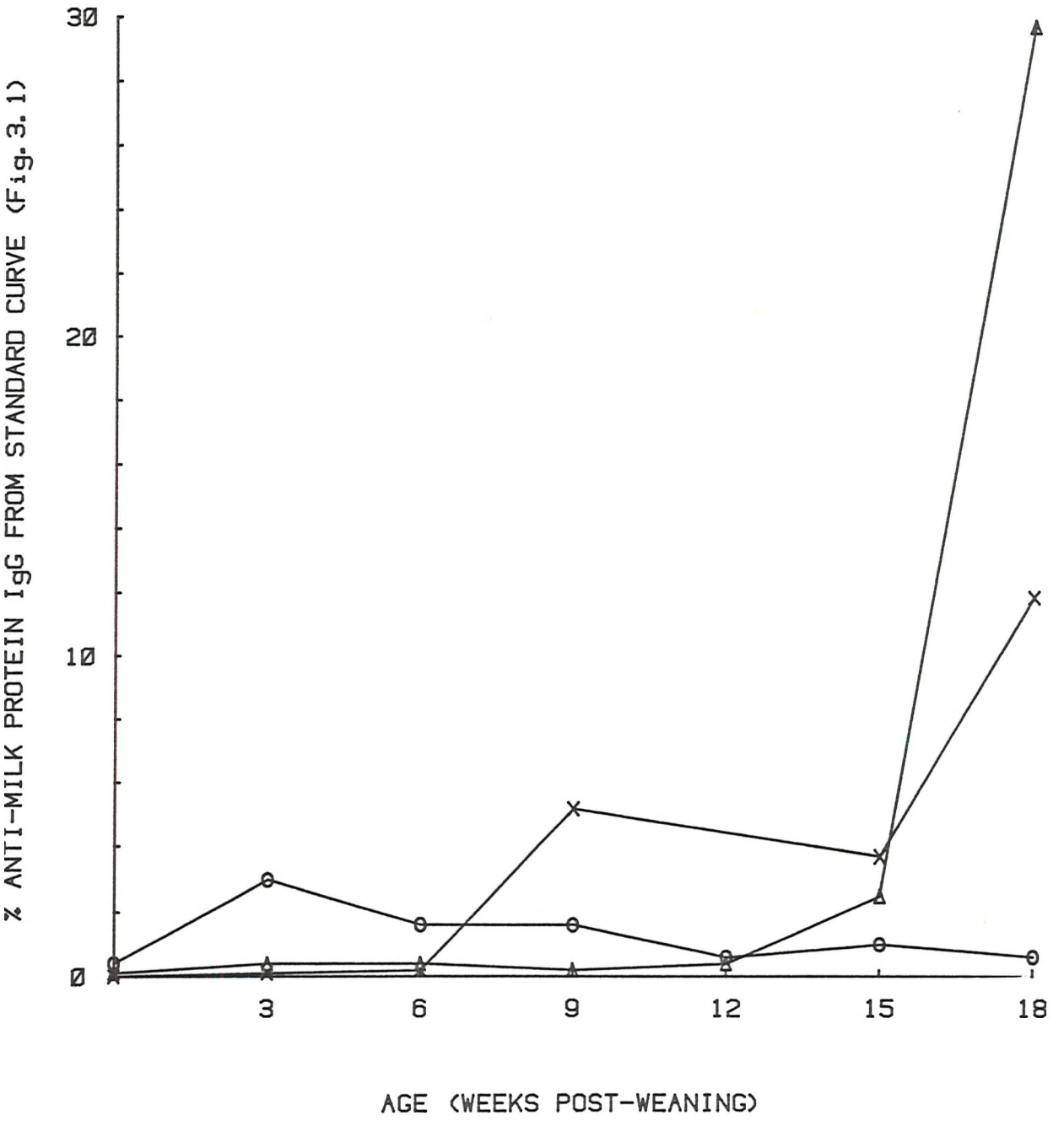


Figure 3.2 ANTI-MILK PROTEIN IgG PRESENT IN THE SERUM OF ANIMALS INTRODUCED TO A DIET CONTAINING 10% DSM AT DIFFERENT AGES. RESULTS EXPRESSED AS A PERCENTAGE OF A STANDARD REFERENCE SERUM (Fig. 3.1).

Discussion

The presence of circulating antimilk protein IgG in the serum increased significantly in the rabbits that were introduced to milk at 6 weeks or 12 weeks post weaning (Group 2 and 3). However, there was no increase in specific serum antibodies over the experimental period in the rabbits fed the diet containing the milk protein from weaning. This indicates that macromolecular absorbtion in the rabbit is not restricted to the gut of the newborn or weanling animal. Protein in an antigenic form continues to cross the mucosal barrier, giving rise to specific circulating antibodies in the mature animal. The lack of specific antibodies in the sera of rabbits in Group 1 may be due either to specific systemic tolerance to the dietary protein or to immune unresponsiveness. Other studies have shown that introducing a dietary protein at weaning to rabbits previously unexposed to the protein results in a dramatic increase in the presence of serum antibodies to that protein *(Gallagher et al., 1982A)*. However, in these experiments the dietary protein investigated made up between 10 and 30% of the diet, whereas in the present study the final concentration of milk protein in the diet was only 3.63% (i.e. 100g per kg diet at 36% crude protein). Therefore, the lack of serum antibodies in Group 1 may reflect the importance of the amount of ingested dietary antigen on the production of circulating antibodies.

The more mature rabbits in Groups 2 and 3 were consuming 7-8g dried skimmed milk a day, whereas the weanling animals may only be consuming 3-5g dried skimmed milk daily and during the first few days after weaning the consumption may have been even lower.

The Daily ingestion of 7-8g soya protein a day has been found to be sufficient to produce a good specific IgG response. However, the consumption of 40 mg of soya per kg diet three times per week was insufficient to stimulate an immune response in adult rabbits *(Pitts, 1983)*.

Farr and Dixon (1959) have also suggested that the antigen concentration is important in initiating antibody synthesis. They also report that antigens differ in their ability to stimulate an antibody response. Dried skimmed milk contains a variety of proteins, including casein, β lactoglobulin, α lactalbumin and BSA. Consequently, a given intake of milk protein per day is an over estimate of the

actual intake of the individual antigen. The table below estimates the quantity of the individual milk proteins ingested by each rabbit daily.

Table 3.4

Individual Milk Protein	Approximate Quantity of Ingested Antigen Per Day (In Grams) (Assuming Rabbits Consume 70g Diet/Day)
Casein	2.17
β Lactaglobulin	0.259
α Lactalbumin	0.092
BSA	0.025

The ability of DSM to produce a systemic immune response will depend on the overall response produced independently by each of the constituent milk proteins.

A second possibility is that the weanling rabbits were unresponsive to the milk protein because it was not recognized as being foreign. Ingestion of maternal milk by the infant and suckling rabbit does not appear to give rise to antibodies against maternal milk protein. This lack of an immune response to maternal milk is not understood, but is possibly due to the close immune relationship between mother and foetus and the early exposure to maternal milk proteins. Another phenomenon which may be important in explaining the unresponsiveness to maternal milk is the precocious secretion of milk from the mammary tissue of the foetus or newborn which has been observed in a number of species *(Cowie et al., 1980)*.

A recent experiment has shown that weanling rabbits have high levels of circulating milk protein, which is probably of maternal origin. However, no circulating antimilk protein antibodies were detected *(Harris & Gibney, unpublished communication)*.

The ability of the mature rabbit to mount an immune response against ingested antigenic dietary protein supports studies by *Korenblat et al., (1968)*. In this study the ingestion of childhood doses of BSA was effective in producing increased amounts of circulating anti-BSA

antibodies in adults who had previously exhibited only low levels of circulating antibody. However, individuals who previously showed no detectable levels of serum anti-BSA antibodies did not produce a rise in serum antibodies when challenged with the supplementary dietary BSA. Thus the observation that antibodies to common dietary proteins are lower in adults than infants may not be due to changes in the gastrointestinal tract that would reduce the amount of antigen entering the circulation, but that acquired immunological tolerance may occur following continuous minimal antigenic challenge *(Goldberg et al., 1971)*.

The permeability of the rabbit gastrointestinal tract is thought to decrease significantly during the first week of life *(Udall, Pang et al, 1981)*, which is possibly facilitated by a factor in the colostrum *(Udall & Colony et al., 1981)*. However, any difference in macromolecula-absorbtion after this period may not be an important factor in determining the immune response to ingested antigens.

Experiment 3b

Introduction

To extend the study of the effect of age on the immune response mounted against an ingested dietary antigen, the following experiment examines the effect of the maternal diet and subsequent tolerance on this response.

The rabbit is a useful non-primate model for this study as both the human and rabbit receive passive immunity exclusively before parturition, although at birth the human infant may be more immunocompetent than the newborn rabbit. Plasma cells containing lgG and lgM and circulating lgM have been identified as early as the fifth month of gestation in the human foetus, which implies the presence of an immunocompetent immune system well before the end of the gestational period *(Van Firth et al., 1965)*. *Rothberg (1969)*, studying the immune system of the premature infant, provides evidence that the onset of antibody synthesis is related to birth and antigenic stimulation rather than gestational age and the initial antibodies detected in the sera of premature infants were to soluble antigenic proteins. The earliest detection of antibodies in the rabbit has been a few hours after birth, but levels are still low one month after birth *(Solomon, 1971)*.

The pattern of antibody production in the rabbit after the ingestion of a soluble protein appears to be similar to the human response. An initial rise in antibodies of the lgM and lgG class occurs followed by a continued increase in lgG and decline in lgM activity *(Rothberg et al., 1967)*. lgG appears in the serum 14-21 days after the ingestion of milk feeding in humans and rabbits *(Rothberg & Farr, 1965A)*.

Previous studies have shown that maternal exposure to a protein will decrease the ability of the offspring to develop serum antibodies to that protein if it is introduced at weaning *(Pathirana et al., 1981)*. The tolerance observed may have either been due to the passive acquisition of maternal antibodies, either from lgG received in utero, via selective transmission across the yolk sac to the foetal circulation *(Brambell, 1958)* (See also Section 1.3.1), or from the lgA that is obtained by the suckling neonate from the colostrum *(Beer et al., 1974)* (See Section 1.1.2(ii)). The presence of circulating maternal antibody has been found to interfere with the induction of active immunity in young animals *(Leiper & Solomon, 1976)*. The interference is believed to be

due to competition for antigen between specific receptors on lymphocytes and antigen combining sites on molecules of circulating antibody from maternal origin. At birth or early in postnatal life, the amount of antibody of maternal origin in the circulation is maximal, reducing the chances of antigen binding with lymphocyte receptors. However, in adult rabbits passive administration of antibodies was found to have no effect on the development of circulating antibodies following the ingestion of a soluble protein *(Rieger et al., 1980)*.

Alternatively, the tolerance may be acquired by small amounts of antigen reaching the young animal either from the maternal breast milk, as a variety of food proteins have been detected in human breast milk *(Gerrard, 1979; Kulangara, 1980)*, or from the preweanling rabbit nibbling the food in the mothers cage. Young rabbits appear to nibble the pelleted maternal diet at 16-18 days of age, which is the period when the young animal is still showing increased absorbtion of intestinal antigens *(Udall, Pang et al., 1981)*. It has been demonstrated that the earlier in life a young animal encounters an antigen the more profound the degree of tolerance that is produced *(Smith, 1961)*.

The effect of the maternal diet on the ability of the offspring to mount an immune response is examined from weaning to maturity.

Experimental Design

8 Female New Zealand White Rabbits were fed a stock diet in which fish was the major protein source (Table 2.1). At 3 months of age 10% DSM (w/w) was added to the diet of four of the female rabbits (Table 2.2). The offpsring remained with their mother until weaning (6 weeks post partum) and were then divided into three groups. Each group consisted of 12 animals, half the weanling rabbits were from dams which had been fed milk protein and half were from dams which had not been exposed to milk. Each group of rabbits was introduced to the diet containing 10% DSM (Table 2.2) at different ages.

Group 1 - Introduced to DSM at weaning (6 weeks post partum)
Group 2 - Introduced to DSM at 6 weeks post weaning
Group 3 - Introduced to DSM at 12 weeks post weaning.

The rabbits had free access to fresh food and water and were housed as outlined in Section 2.6. The experimental procedure was the same as

for Experiment 3a. Blood samples were taken from the marginal ear vein from all rabbits at weaning and then every 3 weeks; body weights were also recorded at 3-weekly intervals.

The serum was collected and used diluted 1:200 with PBST to measure for circulating antimilk protein antibodies as in Experiment 3a. The results were expressed as a percentage of a standard reference serum containing antibodies raised by intramuscular injection against DSM (Fig. 3.1).

Results

The body weight gain of the rabbits is presented in Fig. 3.3 for Group 1, Fig. 3.4 for Group 2 and Fig. 3.5 for Group 3. No significant treatment effects on body weight were found.

The development of antimilk protein lgG was examined by 2 way analysis of variance. A significant increase in specific serum antibodies was observed in each group over the experimental period ($P < 0.001$). There was no overall difference within each group in the development of antibodies by the offspring of dams not fed milk compared to the offspring of dams exposed to milk. Only in Group 3, in which the rabbits were introduced to milk protein at 12 weeks post weaning, was there a significant interaction between specific circulating serum antibodies produced over the experimental period and the maternal dietary history of the rabbits ($P < 0.01$).

Further analysis by the Duncan Multiple Range Test, which is presented in Table 3.5 (Group 1), Table 3.6 (Group 2) and Table 3.7 (Group 3), shows that the offspring of both maternal groups produced an increase in circulating serum antibodies, which appears to be more pronounced in animals from dams which had not been fed milk protein. This difference between the different maternal groups reached significance in the rabbits introduced to the milk diet 12 weeks post weaning (Group 3) ($P < 0.05$).

When the age of introduction to milk was taken as baseline and the production of specific serum antibodies over the next 6 weeks examined a significant increase in serum antibodies to DSM was observed, ($P < 0.001$). The mean results were analysed by the Duncan Multiple Range Test, which demonstrated that in Group 2 and Group 3, a significantly higher level of serum antibodies developed in those rabbits from dams not fed the diet containing milk protein, compared to the

offspring of dams fed milk ($P < 0.05$), which is illustrated in Table 3.8. The difference reaches significance 6 weeks after the introduction to the milk diet in Group 2 and 3 weeks after the introduction to the milk diet in Group 3. However, the presence of significantly greater levels of serum milk antibodies occurred in Group 1 at 3 and 6 weeks in the offspring of dams exposed to milk, compared to the weanlings of dams not exposed to milk ($P < 0.05$).

TABLE 3.5

Mean Values (± S.E.) for Circulating lgG Antibodies to Dietary Cows Milk Protein in Rabbits Introduced to Cows Milk Protein at Weaning (Group 1)

Age of Rabbits (Weeks Post Weaning)	Dams Fed Milk		Dams Not Fed Milk
0	0.156 ± 0.081^{a}	NS	0 ± 0
3	3.155 ± 0.02^{b}	NS	1.55 ± 0.57^{ab}
6	3.42 ± 1.22^{b}	NS	1.72 ± 0.442^{b}
9	8.47 ± 3.13^{b}	NS	10.9 ± 2.80^{c}

The results are expressed as a percentage of a standard reference serum (Fig. 3.1)

a,b,c - Mean values for anti milk protein lgG in a given group without a common superscript differ significantly

NS - Mean values for anti milk protein lgG between groups at a given time do not differ significantly

TABLE 3.6

Mean Values (± S.E.) for Circulating IgG Antibodies to Dietary Cows Milk Protein in Rabbits Introduced to Cows Milk Protein at 6 Weeks Post Weaning (Group 2)

Age of Rabbits (Weeks Post Weaning)	Dams Fed Milk		Dams Not Fed Milk
0	0.083 ± 0.054^{a}	NS	0.075 ± 0.047^{a}
3	0.474 ± 0.311^{ab}	NS	0.389 ± 0.061^{ab}
6	0.659 ± 0.368^{abc}	NS	0.255 ± 0.089^{ab}
9	3.23 ± 1.362^{c}	NS	2.41 ± 0.0843^{bc}
12	2.67 ± 1.066^{bc}	NS	5.10 ± 2.158^{cd}
15	3.59 ± 1.446^{c}	NS	7.97 ± 3.23^{d}

The results are expressed as a percentage of a standard reference serum (Fig. 3.1)

a,b,c - Mean values for anti milk protein IgG in a given group without a common superscript differ significantly ($P < 0.05$)

NS - Mean values for anti milk protein IgG between groups at a given time do not differ significantly

TABLE 3.7

Mean Values (± S.E.) for Circulating lgG Antibodies to Dietary Cows Milk Protein in Rabbits Introduced to Cows Milk Protein at 12 Weeks Post Weaning (Group 3)

Age of Rabbits (Weeks Post Weaning)	Dams Fed Milk		Dams Not Fed Milk
0	0.033 ± 0.033^{a}	NS	0 ± 0
3	0.375 ± 0.061^{bc}	NS	0.393 ± 0.104^{b}
6	0.587 ± 0.196^{bc}	NS	0.29 ± 0.023^{b}
9	0.461 ± 0.063^{bc}	NS	0.251 ± 0.017^{b}
12	0.246 ± 0.058^{b}	NS	0.11 ± 0.067^{b}
15	0.834 ± 0.275^{c}	*	1.84 ± 0.328^{c}
18	2.136 ± 0.369^{d}	*	4.64 ± 0.702^{d}

The results are expressed as a percentage of a standard reference serum (Fig. 3.1)

a,b,c,d - Mean values for anti milk protein lgG in a given group without a common superscript differ significantly ($P < 0.05$)

* - Mean values for anti milk protein lgG between groups at a given time differ significantly ($P < 0.05$)

NS - Mean values for anti milk protein lgG between groups at a given time do not differ significantly

TABLE 3.8

Mean Values (± S.E.) For Circulating IgG Antibodies to Dietary Cows Milk Protein in Rabbits Introduced to Cows Milk Protein at Weaning (Group 1) or at 6 Weeks (Group 2) or 12 Weeks (Group 3) Postweaning

	Group 1			Group 2			Group 3		
Dams Diet	Milk		Non-Milk	Milk		Non-Milk	Milk		Non-Milk
Time after Milk Introduction									
0	0.156 ± 0.081^{a}	NS	0.0 ± 0^{a}	0.659 ± 0.368^{a}	NS	0.255 ± 0.089^{a}	0.246 ± 0.058^{a}	NS	0.11 ± 0.067^{a}
3	3.155 ± 1.02^{b}	*	1.552 ± 0.57^{b}	3.23 ± 1.362^{b}	NS	2.41 ± 0.843^{b}	0.834 ± 0.275^{b}	*	1.84 ± 0.328^{b}
6	3.42 ± 1.22^{b}	*	1.72 ± 0.44^{b}	2.67 ± 1.446^{b}	*	5.10 ± 2.158^{c}	2.136 ± 0.369^{c}	*	4.64 ± 0.702^{c}

The results are expressed as a percentage of a standard reference serum (Fig. 3.1) and compare the effect of maternal exposure to DSM on the values at introduction to cows milk protein and three and six weeks post introduction

a,b - Mean values in any line without a common superscript differ significantly ($P < 0.05$)

NS - Mean values within a given group at a particular time do not differ significantly

* - Mean values within a given group at a particular time differ significantly ($P < 0.05$)

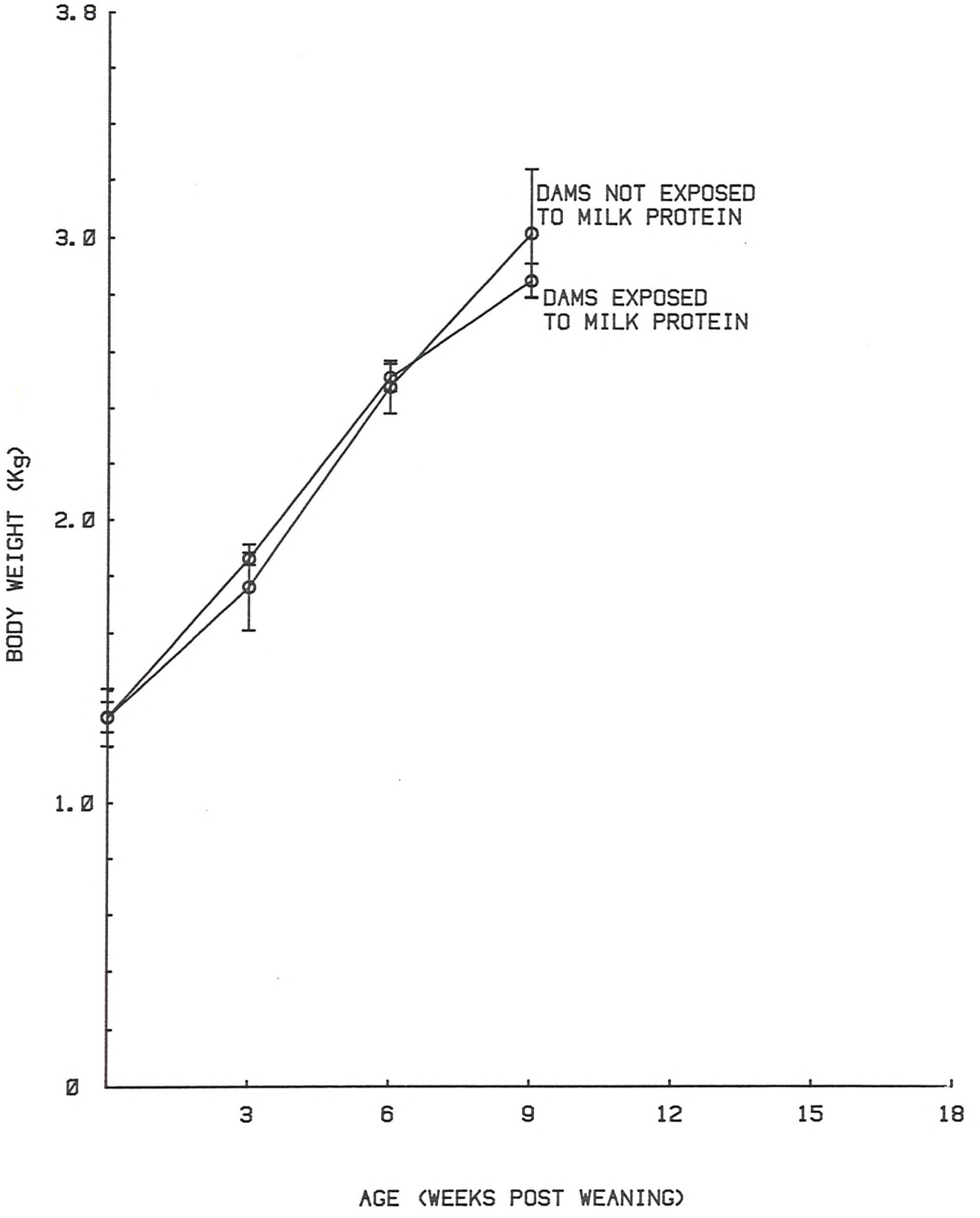


Figure 3.3 MEAN BODY WEIGHT (Kg) + S.E. OF ANIMALS EXPOSED TO MILK PROTEIN FROM WEANING (GROUP 1). OVER THE EXPERIMENTAL PERIOD THERE WAS NO SIGNIFICANT DIFFERENCE IN THE WEIGHT GAIN OF THE TWO GROUPS BY 2 WAY ANALYSIS OF VARIANCE.

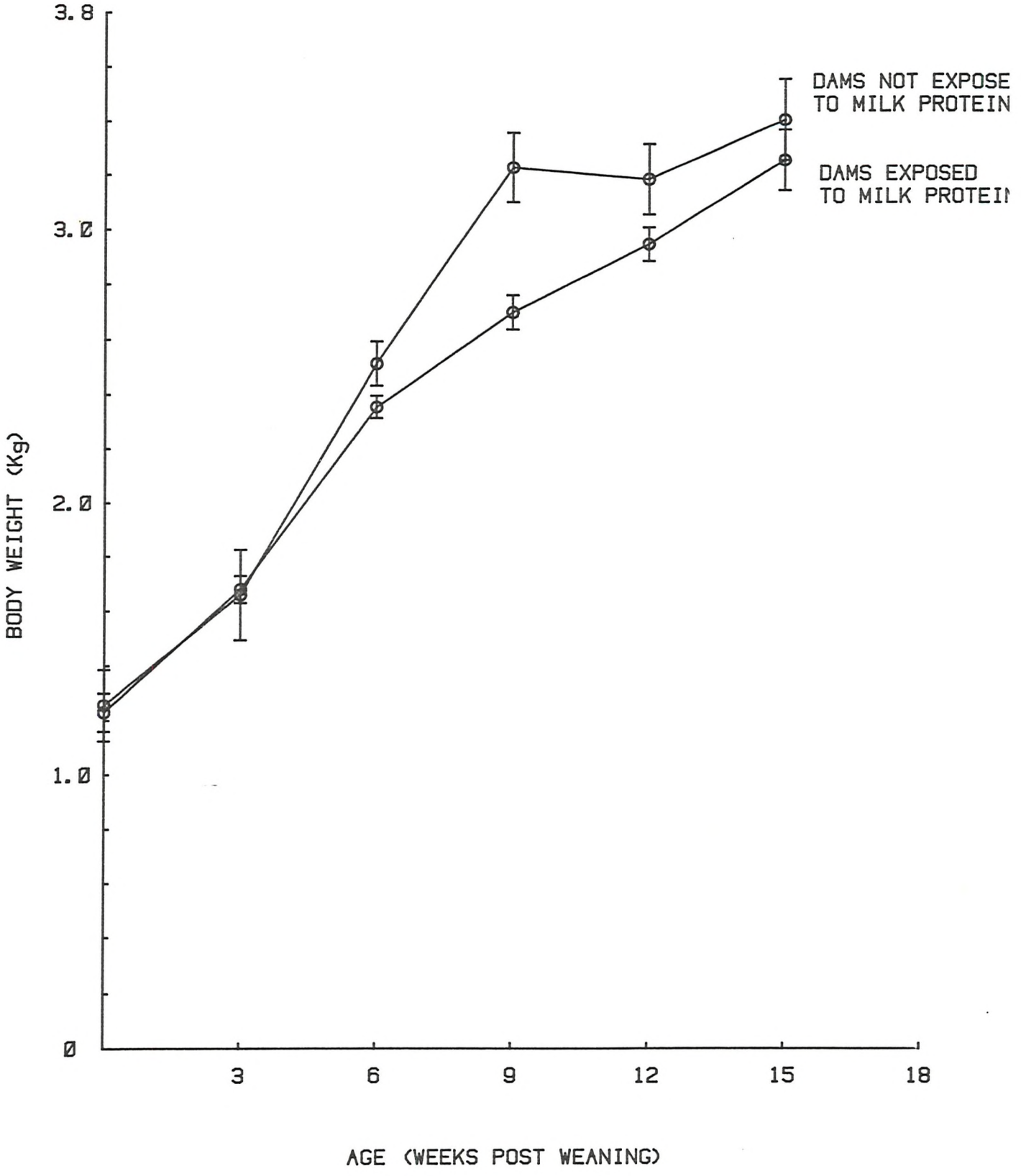


Figure 3.4 MEAN BODY WEIGHT (Kg) + S.E. OF ANIMALS EXPOSED TO MILK PROTEIN 6 WEEKS POST-WEANING (GROUP 2). OVER THE EXPERIMENTAL PERIOD THERE WAS NO SIGNIFICANT DIFFERENCE IN THE WEIGHT GAIN OF THE TWO GROUPS BY 2 WAY ANALYSIS OF VARIANCE.

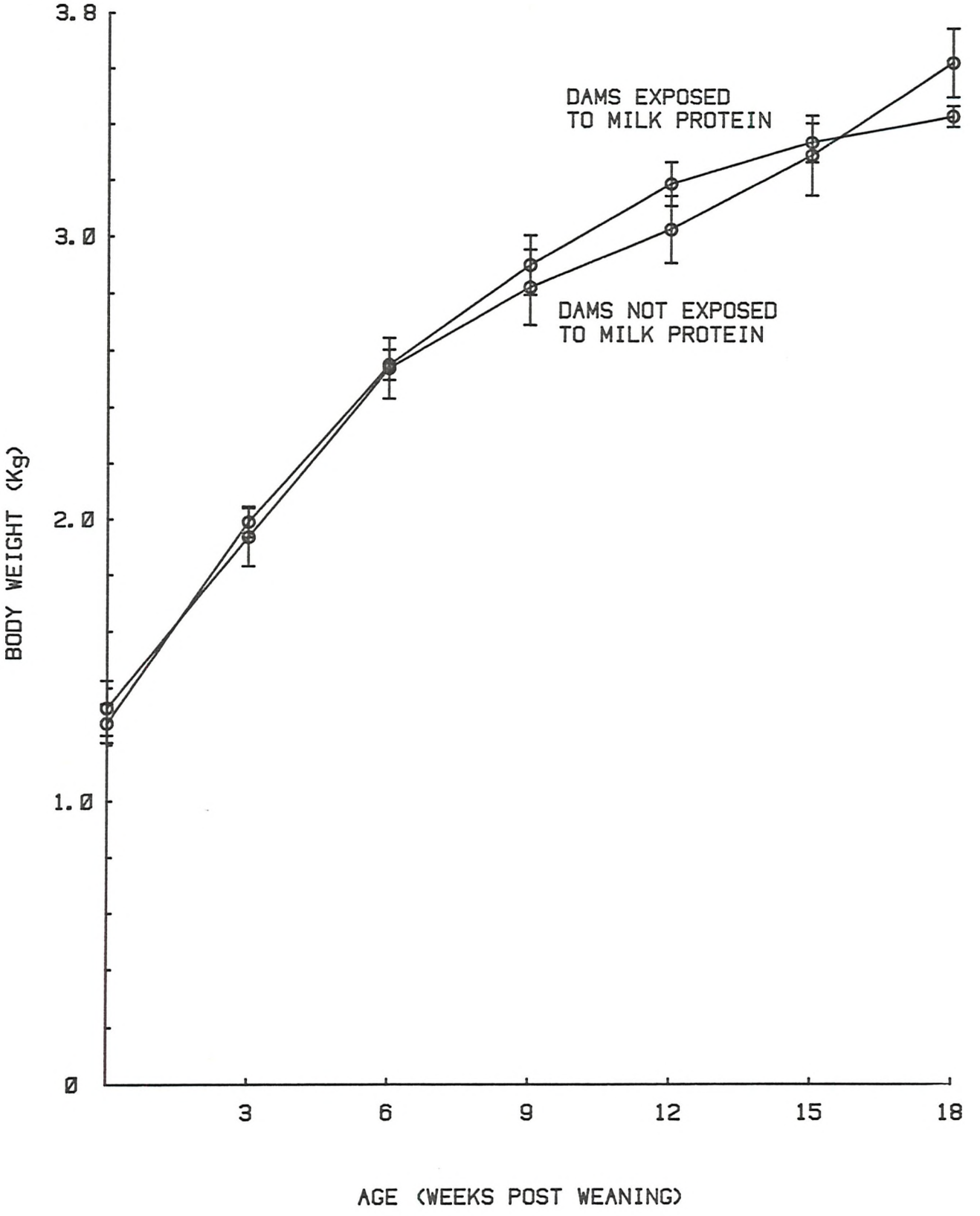


Figure 3.5 MEAN BODY WEIGHT (Kg) + S.E. OF ANIMALS EXPOSED TO MILK PROTEIN 12 WEEKS POST-WEANING (GROUP 3). OVER THE EXPERIMENTAL PERIOD THERE WAS NO SIGNIFICANT DIFFERENCE IN THE WEIGHT GAIN OF THE TWO GROUPS BY 2 WAY ANALYSIS OF VARIANCE.

Discussion

A significant increase in serum antimilk protein antibodies occurred after the introduction of milk protein to the diet at weaning, 6 weeks post weaning and 12 weeks post weaning (Groups 1, 2 and 3).

Maternal exposure to milk protein did not suppress the development of circulating serum antibodies in Group 1, although suppression was observed in Group 3. The rabbits in Group 2 only demonstrated suppressed serum specific antibodies in the offspring from dams exposed to milk protein when the result observed at the age of introduction to the diet containing DSM was taken at the baseline reading. The lack of suppression of antibodies in the serum of the offspring of dams exposed to milk compared with the offspring of dams not exposed to milk when introduced to this protein at weaning does not agree with previous reports *(Pathirana et al., 1981; Gallagher et al., 1982)*. This may be due to the lower level of milk protein in the diet compared to the greater amount of dietary protein present in the diets of the previous studies. These papers also report a lower level of specific circulating antibodies after exposure to a milk based diet, compared with a soya based diet. This anomalous finding is discussed further in Chapter 7.

Examination of the production of serum antibodies by each rabbit individually (Appendix I, Table i & ii) indicates that some animals respond to the ingestion of milk protein by producing serum antibodies, whereas other animals do not. The ability to produce either an immune response or tolerance to a particular dietary antigen may be subject to a certain amount of genetic control. *Chiu and Chien (1972)* found that when the quantity of ingested protein is small, rabbits differed in their ability to produce immune responses when injected with different antigenic components of soyabean and *Kraft and Rothberg (1969)* only stimulated serum antibodies in half the rabbits injected with a low antigen dose. *Rothberg and Farr (1965A)* also found that the ingestion of cows milk protein in rabbits only produced antibodies in the sera of four out of six animals.

In conclusion, the results from Experiments 3a and 3b suggest that the ability to produce circulating serum antibodies is likely to depend on the antigenic properties of an individual protein. The immune response

stimulated by cows milk protein may differ from the response produced by other proteins in weanling rabbits due to the inability of the young animal to recognize the milk protein as foreign. Secondly, the quantity of antigen necessary to result either in tolerance or immunity may be important. Any result obtained using low concentrations of antigen may be dominated by the innate division of animals into responders and non-responders.

The results of both experiments do however indicate that the induction of an immune response to a dietary antigen is not confined to weanling animals, since a significant elevation in mean serum antimilk lgG occurred with antigen introduction at weaning and at 6 or 12 weeks thereafter. Furthermore, the rabbits introduced to milk at 6 and 12 weeks tended to show greater differences, which were statistically significant, between those born of milk fed dams and those born of dams fed on stock diet alone.

CHAPTER 4

THE EFFECT OF PROTEIN LEVEL AND PROTEIN SOURCE ON THE IMMUNOLOGICAL RESPONSE TO DIETARY ANTIGEN

Introduction

The immune response produced by the ingestion of a dietary protein depends not only on the immunological competence of the animal and the presence of maternal antibodies, but also on the physical and chemical properties of the protein.

In the previous chapter, it was suggested that the immunological response to dietary antigen may depend on the amount of protein ingested. *Farr and Dixon (1960)* have confirmed that protein concentration is critical in inducing antibody synthesis following intravenous injection of BSA. However, antigen concentration is not the only criteria for antibody synthesis. *Rothberg and Farr (1965A)* demonstrated that BSA was more effective at stimulating antibodies than α lactalbumin, following the ingestion of skimmed milk, even though the concentration of BSA in milk averaged 0.29 mg/ml whereas the average concentration of α lactalbumin was 1.3 mg/ml. One suggestion for this difference in antigenicity is that the molecular weight of BSA is 60,000, whereas the molecular weight of α lactalbumin is only 16,000.

The frequency with which low levels of antibodies to common food proteins are observed in the serum of healthy individuals suggests that macromolecular absorbtion is a normal physiological process, although raised serum antibodies, especially to egg, milk and wheat may be associated with gastrointestinal diseases *(Bleuminck, 1983)*.

The proficiency by which a macromolecule stimulates the production of serum antibodies depends on the presence of specific antigenic determinants within its structure. Antigenic determinants may be either conformational and are therefore easily destroyed by heating the protein, or they may be a particular sequence of aminoacids within the protein, forming an antigenic determinant which is more stable *(Aas, 1984)*. Antigens can possess determinants which interact with T helper and T suppressor cells and it appears that both determinants exist on the same molecule *(Kölsh, 1984)*.

The following experiment was designed to investigate the formation of specific antibodies in the serum of rabbits after the ingestion of different quantities of dietary protein (Experiment 4a) and the effect of protein source on levels of circulating dietary antigen (Experiment 4b).

Experiment 4a

Experimental Design

Thirty New Zealand White Rabbits were weaned from breeding stock that had only been fed the animal house stock diet in which the protein source was based on fish meal (Table 2.1). The litter from each dam were allocated to two groups of 15 animals each. These 15 rabbits were than randomly allocated into three groups of five. One group of 15 rabbits were fed the ovalbumin enriched diet (Table 2.4). Each subgroup was fed a specific amount of ovalbumin, either 1% (Table 2.4a), 5% (Table 2.4b) or 10% (Table 2.4c) of the total diet (w/w). The second group of 15 rabbits were fed the casein enriched diet (Table 2.3). Each subgroup of 5 rabbits was fed a specific amount of casein either 1% (Table 2.3a), 5% (Table 2.3b) or 10% (Table 2.3c) of the total diet (w/w).

The experimental design is summarized in Fig. 4.1.

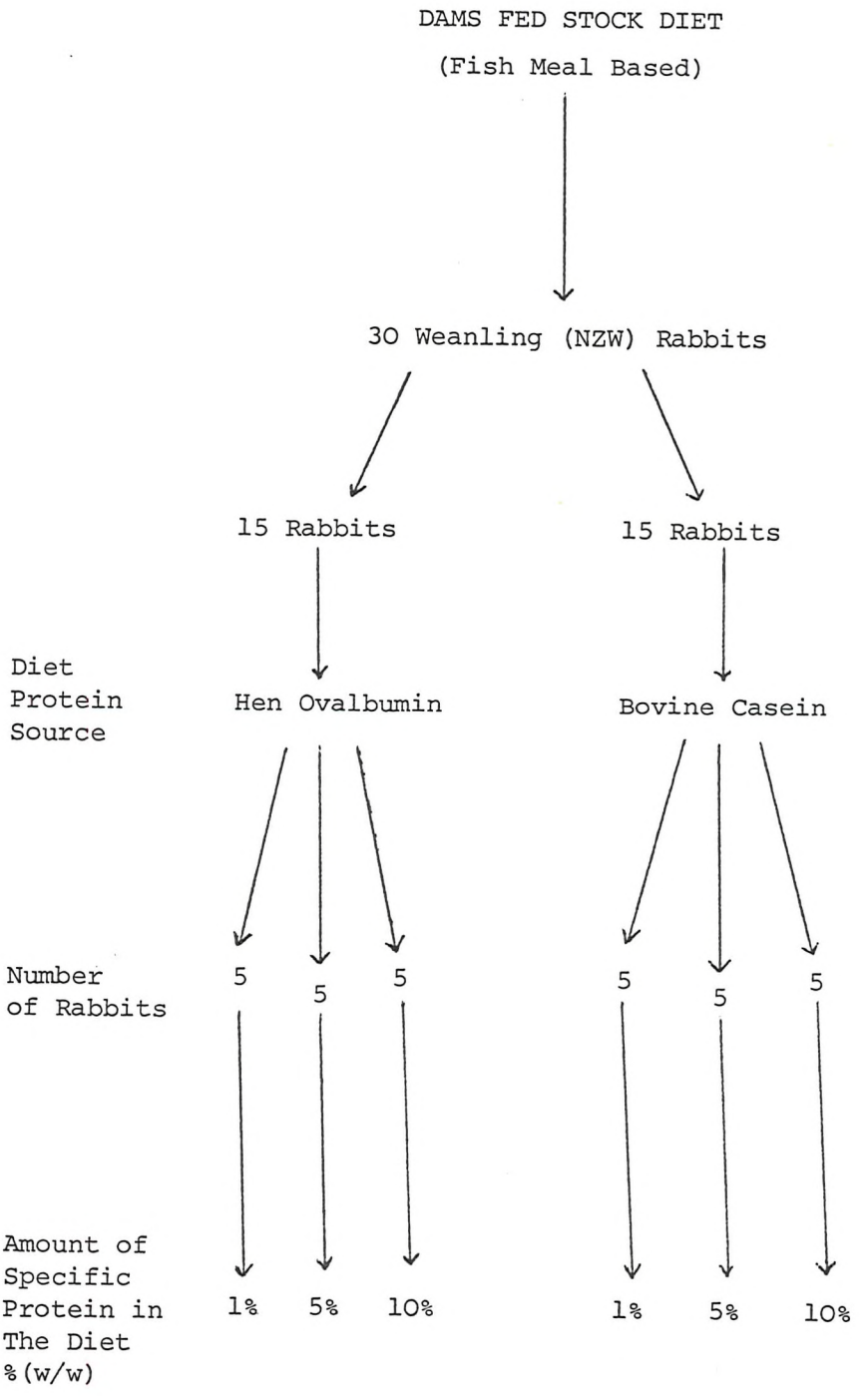
All the experimental rabbits were housed as outlined in Section 2.6 and fed the appropriate diet ad libitum.

Blood samples were taken from the marginal ear vein at weaning and 3 and 6 weeks post weaning. The serum was collected as described in Section 2.6.1 and stored at -22^{o}C.

The analysis of circulating specific antibodies was performed by the ELISA Method (1) (Section 2.1.1). The concentration of ovalbumin and casein used to coat the cuvettes was 10µg protein per ml of coating buffer. The ovalbumin was purchased from Renshaw & Co. Ltd. and the casein was purchased from Sigma Chemicals.

The antibody label employed was goat anti rabbit IgG conjugated to HRP (purchased from Nordic Immunochemicals Laboratories, Tilberg, The Netherlands). The optical density results obtained after the addition of the assay mixture containing O-phenylene diamine were expressed as a percentage of a standard serum sample containing serum antibodies raised against either ovalbumin or casein. The dilution curves of the reference serum from which the percentages were calculated are given in Fig. 4.2 and 4.3 for the casein and ovalbumin reference serum respectively. Details of the immunization regime by which the reference serum was obtained is outlined in Section 2.2.1(i).

FIG. 4.1 EXPERIMENTAL DESIGN



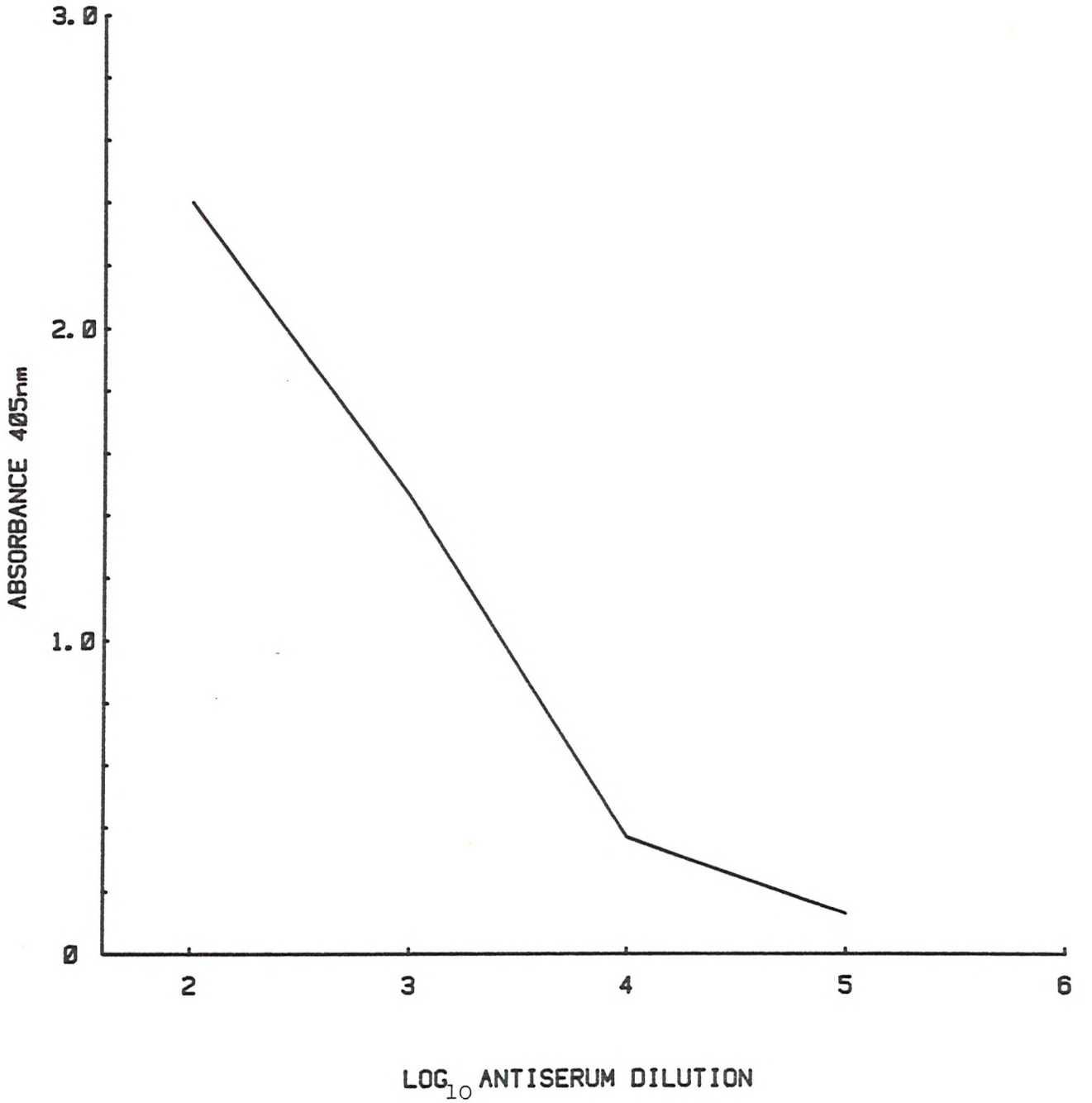


Figure 4.2 STANDARD CURVE. ANTI-CASEIN IgG MEASURED BY ELISA IN SERUM FROM AN ANIMAL IMMUNIZED AGAINST CASEIN.

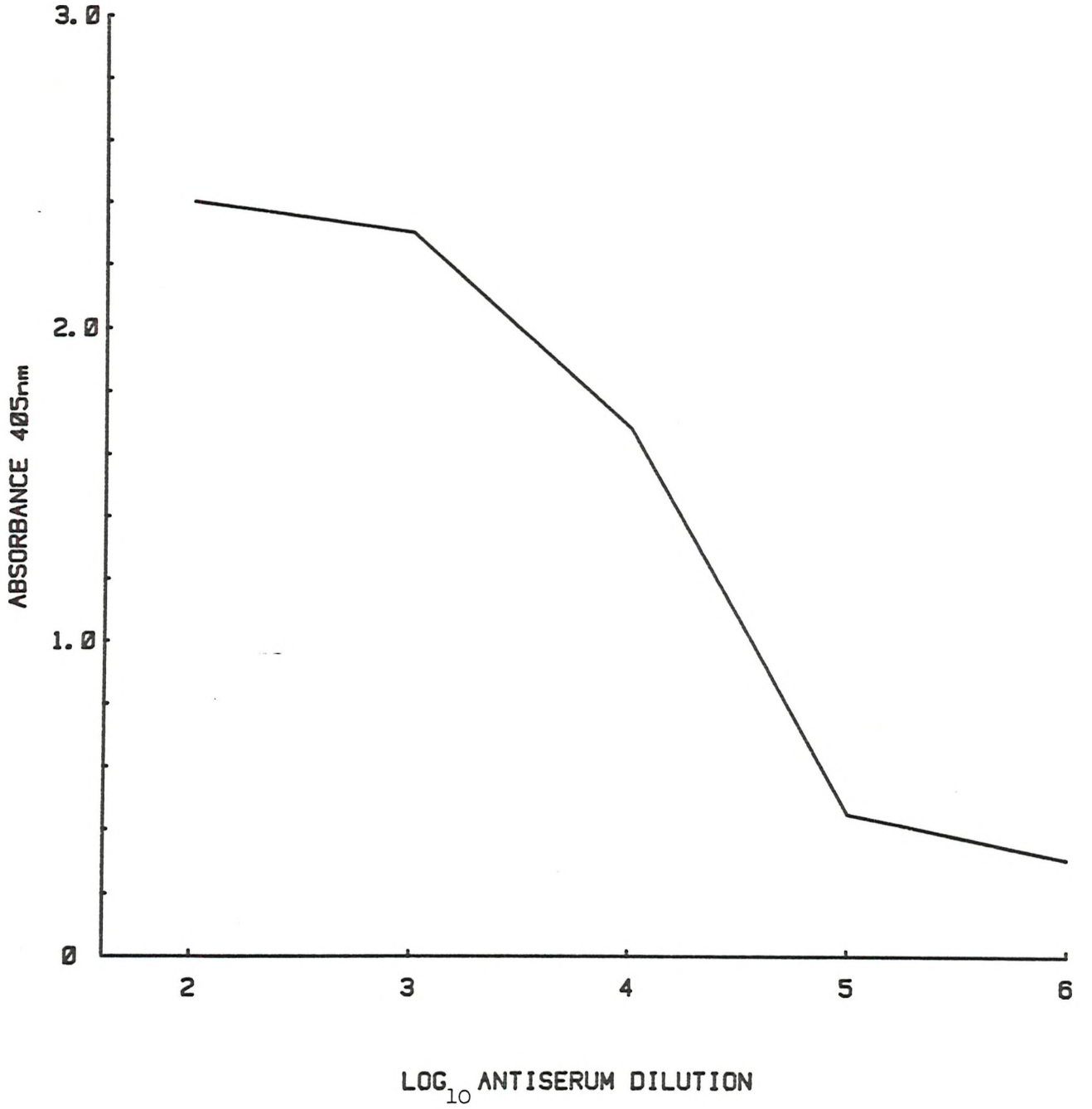


Figure 4.3 STANDARD CURVE. ANTI-OVALBUMIN IgG MEASURED BY ELISA IN SERUM FROM AN ANIMAL IMMUNIZED AGAINST OVALBUMIN.

Results

The mean body weights of the rabbits are presented in Table 4.2, which shows that the body weights of the animals fed either casein or ovalbumin are similar.

The production of serum specific antibodies was examined by two way analysis of variance for each protein, i.e. time and dose effects. Both protein level and time of exposure had significant effects on circulating lgG antibodies to respective antigens ($P < 0.01$ ovalbumin; $P < 0.05$ casein). A significant interaction was observed with ovalbumin fed rabbits ($P < 0.05$) between duration of exposure and level of protein. These results are summarized in Table 4.1.

Table 4.1

Protein Source

Source of Variation	Ovalbumin	Casein
Protein Level	* *	*
Duration of Exposure	* *	*
Interaction	*	NS

~~** P < 0.01~~; * $P < 0.05$; NS - Not Significant

The time and dose effects for each protein were compared by the least significant difference test. The serum specific antibody results following the ingestion of a diet containing casein and ovalbumin are presented in Table 4.3 and 4.4 respectively.

Neither proteins produced a significant increase in serum specific antibodies when the level of protein ingested was 1% or 5% (w/w) of the total diet. However, a significant increase in serum specific antibodies was produced over the experimental period when the quantity of specific protein was increased to 10% (w/w) of the total diet. The increase in serum specific antibodies was more dramatic following the ingestion of a diet containing 10% ovalbumin, compared with a diet containing 10% casein.

Figures 4.4 and 4.5 illustrate the effect of increasing the quantity

of casein or ovalbumin in the diet on the production of serum specific antibodies over the 6 week experimental period. Figure 4.6 compares the protein source and protein level on the production of serum specific antibodies. This illustrates the dramatic effect of increasing the quantity of ovalbumin in the diet compared with casein on the production of serum specific antibodies. However, the large standard error indicates that the production of serum specific antibodies to a particular dietary protein is a very individual response.

TABLE 4.2

Mean Body Weights of the Rabbits (± S.E.)

Age (Weeks post weaning)	Percentage Specific Protein in Diet	
	1% Ovalbumin	1% Casein
0	1.19 ± 0.07	1.32 ± 0.096
3	1.789 ± 0.103	1.85 ± 0.101
6	2.48 ± 0.095	2.50 ± 0.058
	5% Ovalbumin	5% Casein
0	1.29 ± 0.105	1.38 ± 0.062
3	1.88 ± 0.061	1.947± 0.056
6	2.337 ± 0.077	2.506± 0.003
	10% Ovalbumin	10% Casein
0	1.32 ± 0.09	1.21 ± 0.073
3	2.03 ± 0.151	2.18 ± 0.271
6	2.60 ± 0.051	2.43 ± 0.087

Body weight gain within the gtoups fed casein and ovalbumin and between the groups fed casein and ovalbumin did not differ significantly.

TABLE 4.3

Mean Serum Anti-Casein lgG At Different Times After Weaning (% Standard Reference Serum, Fig. 4.2) in Rabbits Fed Different Levels of Dietary Casein

% Casein in The Diet	Age (Weeks Post Weaning)		
	0	3	6
1%	0.495 ± 0.136^{aA}	0.747 ± 0.107^{aAB}	0.767 ± 0.169^{aA}
5%	0.416 ± 0.13^{aA}	0.547 ± 0.209^{aA}	0.496 ± 0.222^{aA}
10%	0.548 ± 0.054^{aA}	0.914 ± 0.269^{abB}	1.33 ± 0.292^{bB}

Pooled S.E.M. = 0.55

a,b - Mean values for any given protein concentration without a common superscript differ significantly ($P < 0.05$).

A,B - Mean values at any given age without a common superscript differ significantly ($P < 0.05$)

TABLE 4.4

Mean Serum Antiovalbumin lgG At Different Times After Weaning (% Standard Reference Serum, Fig. 4.3) in Rabbits Fed Different Levels of Dietary Ovalbumin

% Ovalbumin In The Diet	Age (Weeks Post Weaning)		
	0	3	6
1%	0.236 ± 0.019^{aA}	1.34 ± 0.66^{aA}	4.19 ± 3.17^{aA}
5%	0.205 ± 0.006^{aA}	6.16 ± 2.27^{aA}	5.78 ± 2.66^{aA}
10%	0.165 ± 0.036^{aA}	10.5 ± 1.34^{bA}	47.4 ± 21.7^{cB}

Pooled S.E.M. = 6.34

a,b - Mean values for any given protein concentration without a common superscript differ significantly ($P < 0.05$).

A,B - Mean values at any given age without a common superscript differ significantly ($P < 0.05$).

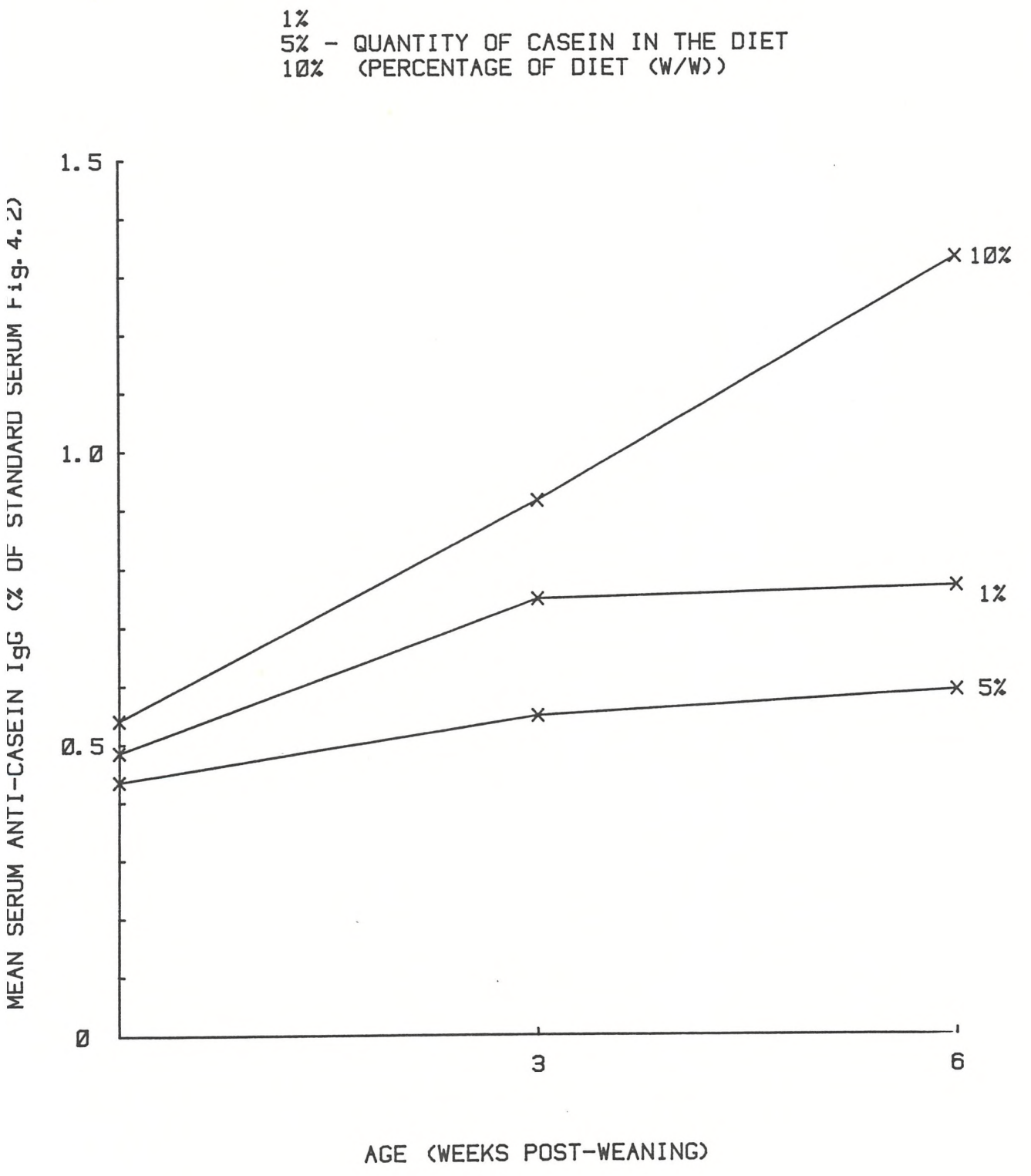


Figure 4.4 EFFECT OF INCREASING THE QUANTITY OF CASEIN ON THE PRODUCTION OF SERUM ANTI-CASEIN IgG.

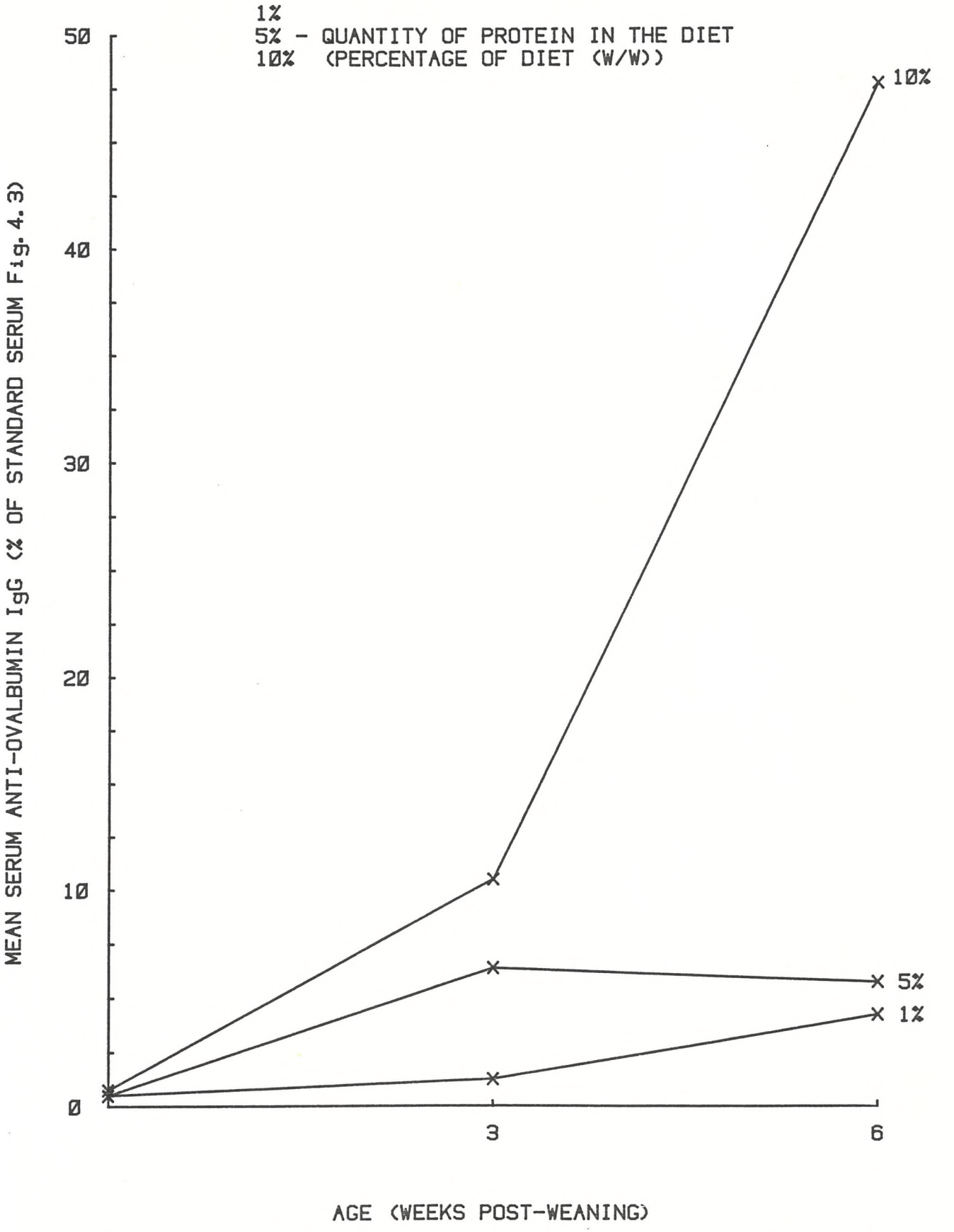


Figure 4.5 EFFECT OF INCREASING THE QUANTITY OF OVALBUMIN ON THE PRODUCTION OF SERUM ANTI-OVALBUMIN IgG.

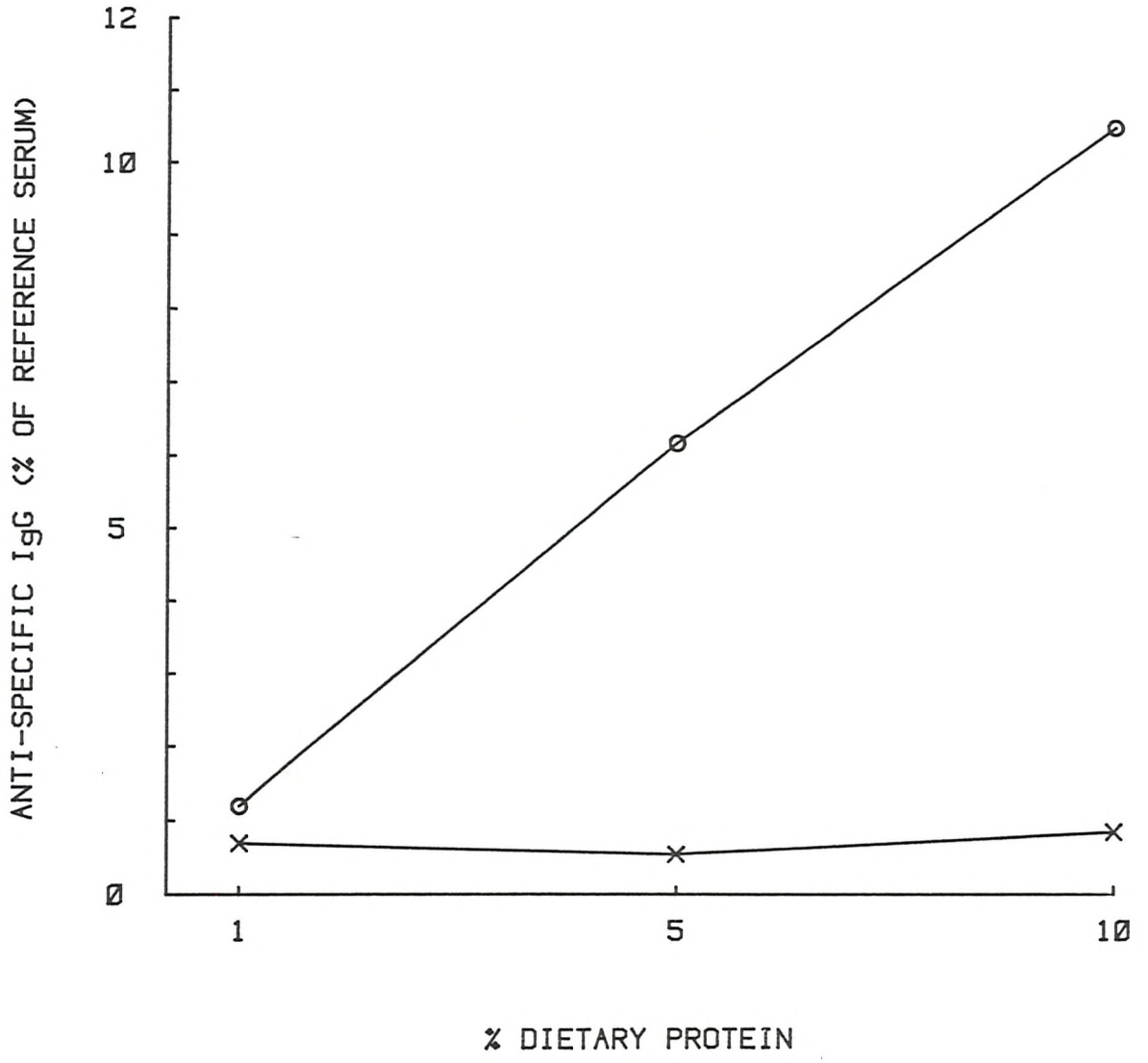


Figure 4.6 A COMPARISON OF THE EFFECT OF DIETARY PROTEIN SOURCE AND DIETARY PROTEIN LEVEL ON CIRCULATING ANTI-OVALBUMIN (O) AND ANTI-CASEIN (X) IgG IN NZW RABBITS THREE WEEKS AFTER ANTIGEN INTRODUCTION.

Discussion

The results demonstrate that the ingestion of ovalbumin is more effective at stimulating circulating antibodies than is the ingestion of a similar amount of casein and that with either protein an increased amount of protein in the diet increases the production of specific serum antibodies. This shows that both the type of protein and the quantity of ingested protein influences the production of specific circulating antibodies.

The ability of an antigen to stimulate an immune response depends on several factors which include the physical and chemical properties of the antigen such as the size and shape of the antigenic molecule, the type of amino acids within the molecule *(Sela, 1969)*, the number of antigenic determinants and their affinity or accessability to the Fc receptor or the B lymphycyte *(Aas, 1984)* and also the presentation of the antigen by the macrophage *(Mosier, 1967; Rosenstriech et al., 1976)*.

The difference in the ability of ovalbumin and casein to stimulate the production of antibodies may be directly related to the quantity of antigenic protein or polypeptide fragments crossing the mucosa of the gastrointestinal tract and entering the circulation. Egg white is a complex mixture of at least 20 separate glycoproteins (See Table 4.5). The major protein which represents about 54% (by weight) of the total egg white protein is ovalbumin *(Bleuminck, 1970)*. It has been suggested that ovalbumin is the major allergen in egg white *(Rapport, 1960; Cambell et al., 1954)*, while others suggest that the positive skin reactions observed after the injection of ovalbumin are to be attributed to ovomucoid protein contaminents in the protein ovalbumin *(Bleuminck & Young, 1969)*. It is possible that the ovalbumin used in the diets does contain traces of other egg white protein and some of these, such as ovomucoid, are known to inhibit the activity of trypsin and chymotrypsin (Table 4.5). This would result in a decreased digestion of ovalbumin and therefore possibly an increase in the absorbtion of undigested or partially digested, and so potentially antigenic, protein.

A recent study in rats on the transmission of proteins from the mother to the foetus has found that ovalbumin is taken up by the maternal gut mucosa and enters the circulation in greater quantities than the other proteins examined. The difference in the uptake of the various proteins did not appear to be related to molecular weight *(Dahl et al 1984)*.

TABLE 4.5

Composition of Egg White

Component	Weight %	Molecular Weight	Remarks
Non Protein Material	8	-	4% Glucose Salt
Ovalbumin	54	45,000	-
Conalbumin	13	77,000	Binds Iron Ions
Ovomucoid	11	31,500	Trypsin Inhibitor
Lysozyme	3.5	15,000	Carbohydrate
Ovomucin	1.5	$\geq$ 100,000	
Flavoprotein	0.8	35,000	Binds Riboflavin
Ovoinhibitor	0.1	78,000	Inhibits trypsin and Chromotrypsin
Avidin	0.05	?	Binds Biotin
Ovoglobulins	8	$\geq$ 100,000	Predominantly serum components

(Bleuminck 1970)

In contrast to ovalbumin, casein appears to precipitate when it enters the stomach and this precipitate is then digested by pepsin. Thus much of the digestion takes place before reaching the intestine. There is little pepsin in the stomach of the newborn so the digestion of casein by the neonate is mainly by trypsin secreted from the pancreas *(McDonald, 1978)*.

Another possible explanation of the increased serum antibodies to ovalbumin compared to casein is that equivalent quantities of potentially antigenic casein and ovalbumin may have reached the circulation, but the weanling rabbit may not have been able to distinguish bovine casein from maternal casein and therefore did not recognize the protein as foreign.

These possibilities are investigated in the following experiment by identifying the amount of ovalbumin and casein present in the serum following the ingestion of a diet containing either 10% casein or 10% ovalbumin.

Experiment 4b

Introduction

The results from the previous experiment (4a) indicate that ovalbumin has a greater capacity to stimulate specific serum antibodies compared with casein, when ingested by the weanling rabbit. This difference in the ability of the two proteins to stimulate the production of specific circulating antibodies may be due to a greater quantity of ovalbumin crossing the gastrointestinal mucosa in a potentially antigenic form when compared with casein. This is supported by the observation that the presence of specific serum antibodies is related to the quantity of protein ingested (Fig. 4.6). This possibility was investigated in this experiment.

Experimental Design

Ten weanling New Zealand White Rabbits were obtained from breeding stock which had been fed a diet containing fish meal as the major protein source (Table 2.1). Five of the weanling rabbits were fed the stock diet with the addition of 10% casein (Table 2.3c) and five animals were fed the stock diet with the addition of 10% ovalbumin (Table 2.4c).

Blood samples were taken from the marginal ear vein at weaning and on days 1, 3, 5 and 14 after weaning.

The serum was collected and stored at -22^{o}C and analysed for specific circulating antigen and antibody by an ELISA Method. The ELISA used to measure the concentration of anti-casein IgG and anti-ovalbumin IgG is described in Section 2.1.1 (Method 1). The concentration of specific protein used to coat the cuvettes was 10 µg protein per ml of coating buffer and the antibody conjugate employed to label the serum antibody was goat anti rabbit IgG (Nordic Immunochemical Laboratories, Tilberg, The Netherlands). The optical density results obtained after the addition of the assay mixture containing O-phenylene diamine as the chromogen were expressed as a percentage of a standard reference serum containing antibodies raised against casein (Fig. 4.2) or ovalbumin (Fig. 4.3) by an intramuscular injection of the protein (Section 2.2.1(i)).

The ELISA employed for measuring specific serum antigen is described in Section 2.3. The serum was diluted 1:10 in coating buffer and added to cuvettes which were coated with purified IgG derived from antiserum against either casein or ovalbumin (Section 2.3.1). The concentration of IgG which was used to coat the cuvettes was 10 µg per ml.

The antigen was detected by the addition of either anticasein lgG conjugated to HRP or antiovalbumin lgG conjugated to HRP, both of which were prepared by the method described in Section 2.3.2. The optical density readings obtained at 405 nm after the addition of the chromogen O-phenylene diamine was related to the quantity of specific antigen in the serum sample. To quantify the amount of antigenic casein or ovalbumin in the serum, standard samples were prepared containing different amounts of the specific antigen and a blank serum (diluted 1:10), which had been obtained from rabbits not exposed to the specific protein. These samples were run in parallel to the test samples and contained concentrations of specific antigen ranging from 100 µg to 10 ng.

The standard curve for casein is given in Fig. 4.7 and for ovalbumin in Fig. 4.8.

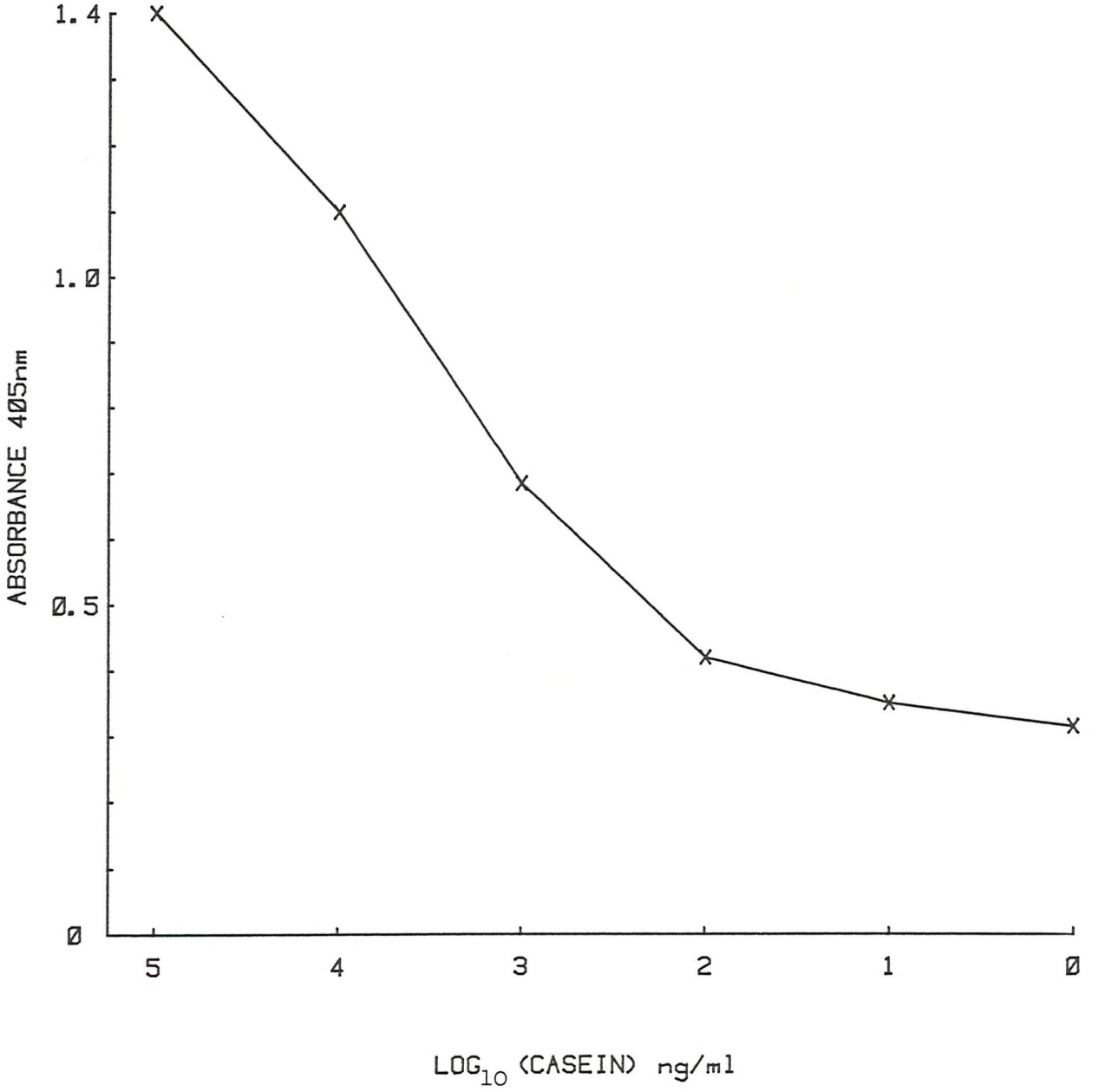


Figure 4.7 STANDARD CURVE RELATING ABSORBANCE AT 405nm TO THE CONCENTRATION OF CASEIN (ng/ml).

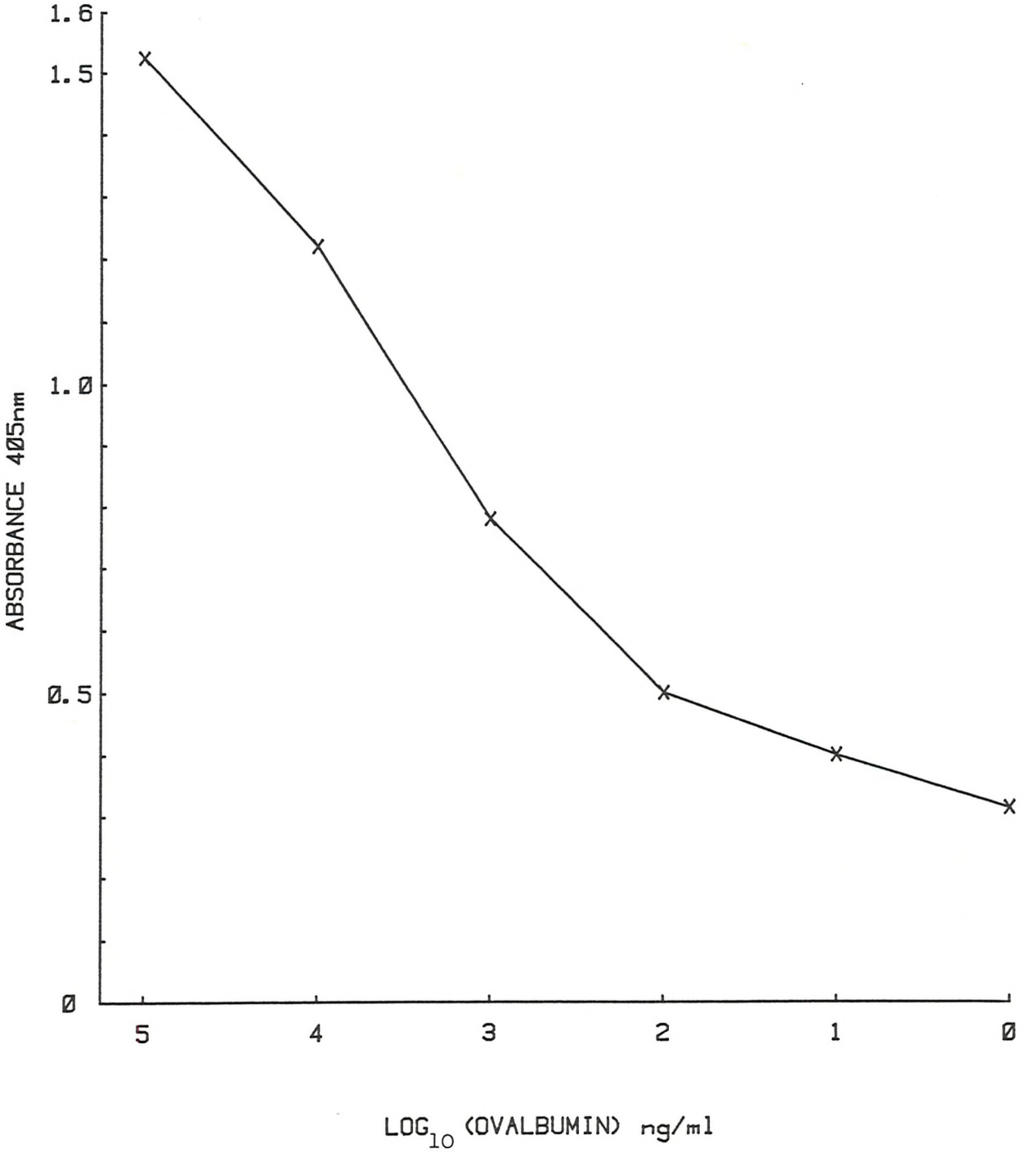


Figure 4.8 STANDARD CURVE RELATING ABSORBANCE AT 405nm TO THE CONCENTRATION OF OVALBUMIN (ng/ml).

Results

Specific Serum Antibodies

The weanling rabbits were fed a diet containing either 10% (w/w) casein or 10% (w/w) ovalbumin over a period of 14 days. During this period, both groups of rabbits produced an increase in specific serum antibodies. Analysis of variance showed that the increase in specific serum antibodies was significant at the 1% level in rabbits fed ovalbumin and at the 5% level in rabbits fed casein.

Comparison of mean values by the Duncans Multiple Range Test (Table 4.6 and 4.7) illustrates that the increase in anti-ovalbumin IgG and anti-casein IgG only reached significance after 14 days.

Circulating Specific Antigen

The concentration of specific antigenic material in the serum was measured over the same time period. The results were analysed by two way analysis of variance, which confirmed that the serum of the rabbits consuming the diet containing ovalbumin contained significantly more antigenic material than the equivalent group containing casein ($P < 0.01$). The concentrations of test antigens in serum for rabbits fed casein or ovalbumin are given in Table 4.8. Comparison of means were made using the Duncans Multiple Range Test. The results show that the quantity of antigenic material reaching the circulation after consuming the diet containing ovalbumin from weaning reached a peak three days after the introduction of the diet (17135 ng/ml) and then decreased to 46 ng/ml of antigenic material 14 days later. The mean values of the concentration of circulating antigenic casein did not differ significantly at any time from the baseline value measured at weaning.

The individual results are presented in Fig. 4.9. The day in which the maximum concentration of specific antigenic material is present in the serum differs with each animal. The individual results produce a mean maximum at day 3, followed by day 5, in the rabbits fed the ovalbumin diet, whereas the maximum mean concentration of antigenic protein in the rabbits fed casein was on day 5 followed by day 14.

TABLE 4.6

Mean Serum Antiovalbumin lgG Antibodies in Rabbits Fed a Diet Containing Ovalbumin

	Days Post Weaning				
	0	1	3	5	14
Mean	0.154[a]	0.02[a]	0.219[a]	0.025[a]	0.859[b]
S.E. ±	0.004	0.003	0.003	0.002	0.075

Results expressed as a percentage of a standard reference serum sample, Fig. 4.3 (± S.E.)

a, b - Mean concentrations of serum antiovalbumin lgG antibodies without a common superscript differ significantly ($P < 0.05$).

TABLE 4.7

Mean Serum Anticasein lgG Antibodies in Rabbits Fed a Diet Containing Casein

	Days Post Weaning				
	0	1	3	5	14
Mean	0.078[a]	0.122[a]	0.099[a]	0.069[a]	0.262[b]
S.E. ±	0.03	0.022	0.0136	0.008	0.0216

Results expressed as a percentage of a standard reference serum sample, Fig. 4.2 (± S.E)

a, b - Mean concentrations of serum anticasein lgG antibodies without a common superscript differ significantly ($P < 0.05$).

TABLE 4.8

Mean Concentration of Antigenic Protein Present in the Serum of Rabbits Fed Diets Containing Either Ovalbumin or Casein

Days Fed The Diet (Age post weaning)	Antigenic Ovalbumin (ng/ml)		Antigenic Casein (ng/ml)
0	22 ± 19.59[a]	NS	5.0 ± 0[a]
1	8300 ± 5564[ab]	NS	59 ± 34.1[a]
3	17135 ± 10623[b]	*	102 ± 34.3[a]
5	13324 ± 5756[ab]	NS	1192 ± 961[a]
14	46 ± 22.0[a]	NS	597 ± 195[a]

, NS - Statistical comparison of the mean antigenic concentrations in animals fed ovalbumin or casein. (: < 0.05; NS: Not Significant)

a, b - Mean values within each dietary group without a common superscript differ significantly ($P < 0.05$).

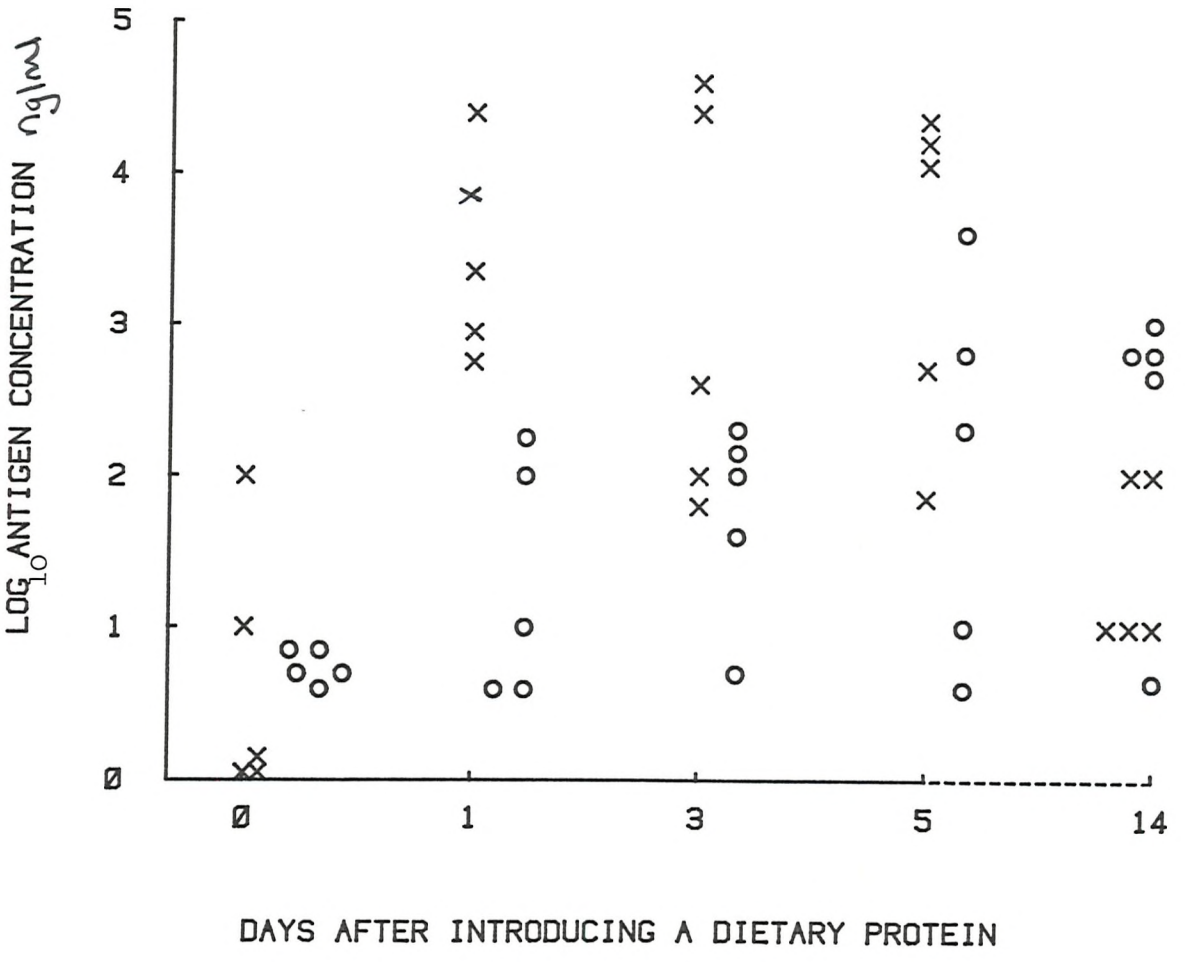


Figure 4.9 VALUES FOR CIRCULATING ANTIGEN LEVELS IN INDIVIDUAL RABBITS FED OVALBUMIN (X) OR CASEIN (O) AT DIFFERENT TIMES AFTER WEANING.

Discussion

The concentration of specific serum antibodies did not increase significantly until 14 days after the introduction of the experimental diet. This coincides with the length of time required for the production of serum specific antibodies observed in other studies *(Rothberg & Farr, 1965A)*. As was observed in Experiment 4a, ovalbumin induced a greater immune response than casein.

The difference in serum antibodies produced by the ingestion of casein and ovalbumin may be explained by the finding that significantly greater quantities of ovalbumin crossed the gastrointestinal barrier and entered the circulation in a form which was able to bind to specific anti lgG antibodies, thus indicating that the quantity of antigenic protein that crosses the gastrointestinal mucosa and reaches the circulation is critical for the production of increased concentrations of serum antibodies.

The concentration of ovalbumin that was detected in an antigenic form was maximal in the serum of rabbits 3 days after the introduction of the diet containing ovalbumin. This declined such that by day 14 only minimal concentrations of the specific antigen were detected. This decline parallels the rise in specific serum antibodies. Hence, the decline in antigen concentration may be due to the formation of immune complexes which would result in the rapid removal of the antigen from the circulation. Complexes containing two or more lgG molecules are quickly removed from the circulation largely by the reticuloendothelial system *(Mannik & Arend, 1971)*. However, when the quantity of antigen is in excess the formation of small soluble complexes occurs *(Nydegger, 1979)*. The decline in the concentration of antigen in the circulation may be due to its binding with the developing immunoglobulin response, thus reducing its detection in ELISA. Alternatively, the systemic immune response detected at day 14 may have coincided with a local gut secretory lgA response, thus reducing further antigen crossing the gastrointestinal barrier.

The small antibody response to ingested casein appeared to be low due to levels of antigenic casein crossing the gastrointestinal mucosa and reaching the circulation. The low levels of antigenic casein found at weaning, contrast with comparable values found for antigenic whole milk protein at weaning (Table 7.9).

The antigenic protein present in the serum was not identified in this study, thus leaving open the possibility of circulating anti-idiotypic antibodies. The presence of idiotypic antibodies against the antibodies raised against casein or ovalbumin would affect the result for serum antigen.

CHAPTER 5

THE IMMUNOLOGICAL RESPONSE TO DIETARY ANTIGEN IN THE MOUSE AND THE EFFECT OF AGE AT ANTIGEN INTRODUCTION

Introduction

In contrast to the rabbit and human, which acquire passive immunity in utero, the mouse receives passive immunity both pre and post natally, although the majority of maternal antibodies are received by selective uptake of IgG from the maternal milk. The transmission of passive immunity has been well reviewed by *Brambell, (1970) and Billington & Wild (1979)*, and has also been discussed in Section 1.3.

Maternal antibodies in the rabbit are exclusively transmitted via the yolk sac during the second half of pregnancy, i.e. from about the 15th day of gestation. At this time, the circulation becomes channelled through vitelline arteries via the yolk sac to the umbilical cord and thence to the the foetus *(Schlamowitz, 1976)*. The antibodies in the foetal circulation rise from about the 22nd day of gestation to full term reaching a maximum on the 27th day, when the foetal antibody titre equals or exceeds the titre in the maternal serum. The transmission appears to be receptor mediated and selective for rabbit IgG *(Wild, 1981B)*.

The importance of the maternal antibodies transmitted before parturition in the mouse is not fully understood. *Carretti and Ovary (1969)* reported that a certain amount of maternal IgG is transmitted before birth in the mouse, and *Morphis and Gitlin (1970)* described a maternofoetal IgG transport mechanism which becomes fully active between the eleventh and fifteenth day of gestation. A more recent study on the presence of maternal immunoglobulins in the mouse suggests that transmission of antibodies from mother to young begins relatively early in the second half of gestation, involving IgG of the isotypes γ_1, γ_{2a} and γ_{2b}. However, in contrast to the high concentration of maternal antibodies in the newborn rabbit, the antibody titre in the newborn mouse is only 10% of that in the maternal serum. This titre was found to fall one day after birth, causing the neonatal mouse to have substantially lower levels of immunoglobulin than was observed in the last few days of foetal life *(Appelby & Catty, 1983)*. This initial fall observed in the neonatal mouse following birth may be the result of a number of factors, such as the immature IgG transport mechanism in the neonatal gut or low levels of immunoglobulin in the first colostral secretions. Serum loss or other trauma associated with parturition

may induce transient hypogammaglobulinaemia, which was observed to persist for 1-2 days until the neonate was able to actively concentrate maternal lgG from the maternal milk.

The mechanism of postnatal absorbtion of lgG has been extensively studied in the rat, the process being mediated by Fc receptors located on the surface of cells lining the small intestine of the neonate *(Rodewald, 1976)*. The concentration of maternal lgG in the serum of the neonate reaches a peak at day 10, which is maintained until day 16. At this time the serum antibody titre of the neonate may reach twice that of the maternal serum, but declines after day 16 due to the cessation of the selective uptake of lgG from the maternal milk *(Halliday, 1959)*.

In the mouse, lgG_1 and lgG_{2a} are preferentially taken up into the neonatal circulation *(Appelby & Catty, 1983)* - this may be because they are the isotypes which have an increased rate of synthesis in the adult or a higher affinity for the Fc receptors compared to lgG_{2b} and lgG_3.

Because of the efficient transport of lgG across the gut wall there is no demand for a high level of lgG in the milk, therefore the predominant immunoglobulin in the milk is lgA. lgA has the primary role of protecing the neonate from the invasion of antigenic material across the gut wall *(Tomasi & Bienenstock, 1968)*. *Weisz-Carrington et al., (1984)* have shown that the epethelial cells of the mouse mammary gland selectively bind and transport lgA and this capacity is hormone dependent.

Given these differences in the acquisition of immune status by rabbits and mice, it was decided to investigate the immunological response to dietary protein in mice. Three experiments were carried out. The immunological response to ovalbumin produced in the weanling mouse and the effect of maternal exposure was examined in Experiment 5a. The decline in maternally derived antibodies to dietary protein was investigated in 5b and the effect of age on these phenomenon were examined in Experiment 5c.

Experiment 5a

Experimental Design

12 female weanling albino mice were divided into two groups, 6 of the female mice were fed a control diet which did not contain ovalbumin (Table 2.6) and 6 were fed a diet which contained ovalbumin as the major protein source (Table 2.7). At six weeks the mice were mated and throughout pregnancy and lactation they were maintained on the above allocated diet.

The experimental design is illustrated in Fig. 5.1.

The mice from each group were killed by decapitation. Blood was collected and allowed to clot for 1-2 hours at 37°C. The serum was separated by centrifugation and removed using a pasteur pipette. It was stored in 200 µl aliquots at -22°C for subsequent determination of anti-ovalbumin IgG by the ELISA method as outlined in Section 2.1.1, method 1.

The serum was diluted 1:20 with PBST and added to cuvettes which had been coated with 10 µg per ml ovalbumin (Renshaw & Co., Ltd.). The antibody conjugate used to label the anti-ovalbumin IgG in the serum was Rabbit anti Mouse IgG conjugated to Horse Radish Peroxidase (Nordic Immunochemical Laboratories). The results were expressed as the optical density recorded at 405 nm after the addition of the assay mixture containing O-phenylene diamine as the colour reagent.

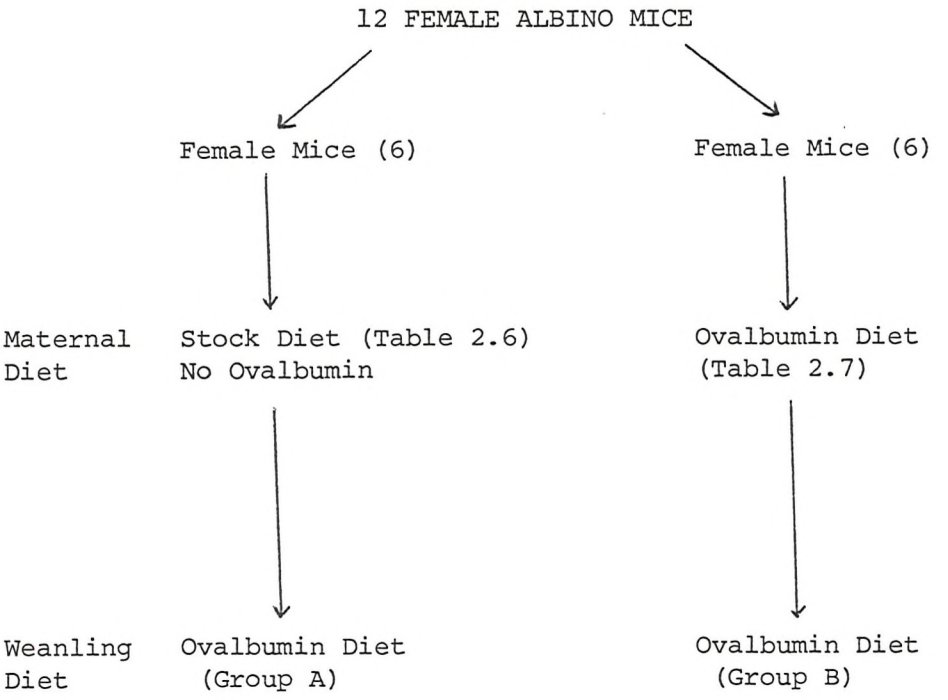
Due to the problems in immunizing and obtaining sufficient antisera from mice, the results were not converted to a percentage of a standard reference serum.

Results

The results were analysed by 2-way analysis of variance. There was a significant interaction between the maternal diet of the offspring and the pattern of antibody production over the experimental period ($P < 0.01$). However, there was no general increase in the development of antibodies with increasing time or a significant difference between the overall presence of circulating specific antibodies between the two groups.

Mean values for anti-ovalbumin production was compared both between and within the two groups by the Duncan Multiple Range Test at the 5%

FIG. 5.1 EXPERIMENTAL DESIGN



15 mice from each group were killed by decapitation at 0,1,2,3,4 and 6 weeks post weaning and the serum was measured for antiovalbumin lgG

significant level; the results are presented in Table 5.1. The offspring from maternal stock which had not been exposed to ovalbumin (Group A) produced a significant increase in circulation anti-ovalbumin lgG during the first week after being weaned onto the diet containing ovalbumin, and this level of serum specific antibodies observed at 1 week post weaning was maintained throughout the experimental period (6 weeks post-weaning).

In contrast, the offspring from maternal breeding stock which had been exposed to ovalbumin (Group B) had a significantly greater concentration of circulating anti-ovalbumin lgG at weaning than was observed during the following 6 weeks. During this latter period, the concentration of circulating anti-ovalbumin lgG remained relatively constant. Thus at weaning the level of specific serum antibodies in Groups A and B differed significantly, subsequently this difference disappeared.

The serum was diluted by a factor of 20 with PBST to enable the majority of the optical density readings to fall on the linear part of the sigmoidal curve that is produced when antibody concentration is plotted against absorbance. The dilution factor is therefore 1/100 of that used to measure specific circulating antibodies in the serum of rabbits after the ingestion of a dietary protein.

TABLE 5.1

Mean Serum Anti Ovalbumin lgG in Mice
Exposed to Ovalbumin at Weaning

(Serum Diluted 1:20)

Age (Weeks Post Weaning)	Group A Maternal Breeding Stock not Exposed to Ovalbumin		Group B Maternal Breeding Stock Exposed to Ovalbumin
0	0.18 ± 0.005^{a}	*	1.29 ± 0.27^{a}
1	0.72 ± 0.106^{b}	NS	0.66 ± 0.06^{b}
2	1.02 ± 0.219^{b}	NS	0.90 ± 0.171^{b}
3	0.84 ± 0.215^{b}	NS	0.57 ± 0.005^{b}
4	1.03 ± 0.043^{b}	NS	0.92 ± 0.25^{b}
6	0.94 ± 0.398^{b}	NS	0.84 ± 0.08^{b}

* - Serum antiovalbumin lgG differ significantly between the two groups at a given age ($P < 0.05$)

NS - No significant difference in serum anti ovalbumin lgG at a given age between the two groups

a,b - Mean values within a given dietary group without a common superscript differ significantly ($P < 0.05$)

Experiment 5b

Experimental Design

12 female albino mice were fed a diet containing ovalbumin (Table 2.7). The mice were maintained on this diet throughout pregnancy and lactation.

At 21 days post-partum the offspring were weaned onto laboratory mouse stock diet (Table 2.6). Fifteen of the offspring were killed prior to weaning, on day 17 post partum and 15 were killed at weaning (21 days post partum). The remaining mice were killed in groups of 15 at 5, 12 and 20 days post weaning.

The mice were killed by decapitation and the blood collected. The blood from three mice was pooled to make one sample. The serum was removed and measured for anti-ovalbumin lgG by the ELISA method, described in Section 2.1.1, method 1. The cuvettes were coated with ovalbumin at a concentration of 10 µg/ml and the antibody used to label the serum anti-ovalbumin lgG was Rabbit anti Mouse lgG conjugated to HRP (Nordic Immunochemical Laboratories). The results were expressed as the optical density reading at 405 nm recorded after the addition of O-phenylene diamine as the chromogen.

A control group of 15 mice in which the breeding stock had not been exposed to ovalbumin but fed a laboratory stock diet was sacrificed at weaning and the serum measured for anti-ovalbumin lgG.

The serum of all the mice was diluted in PBST 1:20.

Results

A significant decrease in serum anti-ovalbumin lgG was observed in the mice with increasing age ($P < 0.001$), illustrated in Table 5.2. The mean values were compared by the Duncans Multiple Range Test, which confirmed that serum antibodies declined from 17 to 21 days of age to lower levels post weaning. The continued decline in the optical density values from 5 days post weaning to 20 days post weaning was not significant, and by 20 days post weaning, the antibody result had not declined to the value of the control group, indicating the presence of anti-ovalbumin lgG.

The value of 0.446 ± 0.013 observed in the control group suggests the presence of a certain amount of non specific binding.

TABLE 5.2

Mean Anti Ovalbumin lgG (± S.E.) in Weanling Mice (Bred from Mothers Exposed to Ovalbumin) Fed a Stock Mouse Diet

(Serum Diluted 1:20)

Age of Mice (Days Post Partum)	Mean Serum Antiovalbumin lgG ± S.E.
17	1.513^{a} ± 0.06
21	1.390^{a} ± 0.079
26 (5 days post weaning)	1.04^{b} ± 0.083
33 (12 days post weaning)	0.924^{b} ± 0.204
41 (20 days post weaning)	0.80^{b} ± 0.046

a,b - Mean values without a common superscript differ significantly ($P < 0.05$)

Control Stock Fed Mice (No Maternal Exposure to Ovalbumin)	
21 Days of Age	0.446 ± 0.013

Discussion of Experiments 5a and 5b

The results from experiment 5a show that the immune response produced in the weanling mouse following the ingestion of a novel dietary protein (Group A) is similar to the serum antibody response observed in weanling rabbits, both animals produce an increase in specific serum antibodies. The serum antibodies had risen by 1 week post weaning in the mice and the level was maintained for the following 5 weeks. In contrast, mice from maternal breeding stock which had been exposed to ovalbumin (Group B) had elevated levels of serum anti-ovalbumin lgG at weaning. This contrasts with studies using rabbits in that the offspring of these species weaned from dams given a test protein do not show high levels of antibody to the test antigen at weaning.

Two possibilities exist to account for the presence of serum antibodies in Group B at weaning. Firstly, antibody synthesis may have been stimulated in the offspring by small amounts of ingested antigen reaching the circulation either in utero *(Dahl et al., 1984)* or via the maternal milk *(Kalangara, 1980)*, or from the suckling mouse nibbling the mothers food. However, this is unlikely to account for all the detected serum antibody since lgG is not actively synthesised by the young mouse until a few weeks after birth *(Appelby & Catty, 1983)*. The second possibility is that the antibodies detected in the serum of the mice at weaning is of maternal origin.

Leiper and Solomon (1974) found that the presence of maternal antibody interfered with the plaque forming cell (PFC) response in 10-day old rabbits. A more recent study also reports that infant mice from mothers which had been immunized with sheep red blood cells were unable to mount a lgM or lgG PFC response to this antigen, which the authors attributed to the presence of maternal antibodies *(Yamaguchi et al., 1983)*.

The mechanism of a suppressed immune response in the neonate by the presence of maternal antibodies may be due to feedback suppression by the excess maternal antibody preventing proliferation of antibody producing cells by the neonate, or alternatively, the circulating maternal antibody may bind with antigen before antigen can stimulate the appropriate neonatal lymphocyte *(Leiper & Solomon, 1976)*.

The decline in the serum anti-ovalbumin lgG with age in the mice of experiment 5b possibly represents the decline in maternal antibodies.

Serum antibodies were maximal in the mice killed prior to weaning at 17 days of age, which is about the time when the gut of the neonatal mouse ceases to take up maternal IgG from the colostrum. The concentration of serum antibodies at 20 days was still significantly higher than at 26 days (5 days post weaning). However, the decline in serum antibodies from 5 days post weaning to that observed at 12 and 20 days after weaning was not significant and when these values were compared to a control group of mice where the maternal diet had not contained ovalbumin there still appeared to be antibodies to ovalbumin in the serum of these mice.

The studies by *Appelby and Catty (1983)* confirm that maternal antibody is still present in the serum of the neonate in large amounts at 20-21 days after birth, the age at which the mice in experiment 5a and experiment 5b were weaned. The presence of maternal antibody was found to persist at a titre of less than 10% of the maternal titre 27 days after birth, and in some cases traces of maternal antibody were detected as late as 30 to 40 days after birth. The length of time maternal antibody persists in the serum of the offspring is likely to depend on the route and dose of maternal immunization *(Yamamguchi et al., 1983)*, the type of antigen and the route by which the offspring acquires passive immunity.

Passive immunity in the rabbit is received in utero and not after birth. This is perhaps why the immune response by the neonate is influenced only by maternal antibodies up to 10 days post partum *(Leiper & Solomon, 1974)*, whereas in the mouse, maternal antibodies are received up to 16 days after parturition so they are able to influence the immune response of the neonate for a longer period of time after birth. Therefore, the antibodies in Group B at weaning are probably due to maternal antibodies which, from experiment 3b, seem to slowly decline. This may coincide with the production of specific serum antibodies by the young animal. Any unresponsiveness exhibited by the neonate may be masked by the presence of maternal antibodies.

The result obtained from the control group in experiment 5b indicates the presence of a certain amount of non-specific binding.

Experiment 5c

Experimental Design

12 female weanling albino mice were divided into two groups, 6 of the female mice were fed a diet based on soya protein (Table 2.8a) and 6 were fed a diet containing soya and ovalbumin (Table 2.8b). At 6 weeks of age, the mice were mated and 60 offspring were weaned from the maternal stock which had not been fed ovalbumin (Group X) and 60 offspring were weaned from maternal stock which had been fed ovalbumin (Group Y). Each group of 60 weanling mice (Group X and Group Y) were divided into 4 subgroups, each consisting of 15 mice.

15 mice from Group X and Group Y were killed at weaning while all the remaining mice were introduced to the diet containing ovalbumin (Table 2.8b) at the following ages.

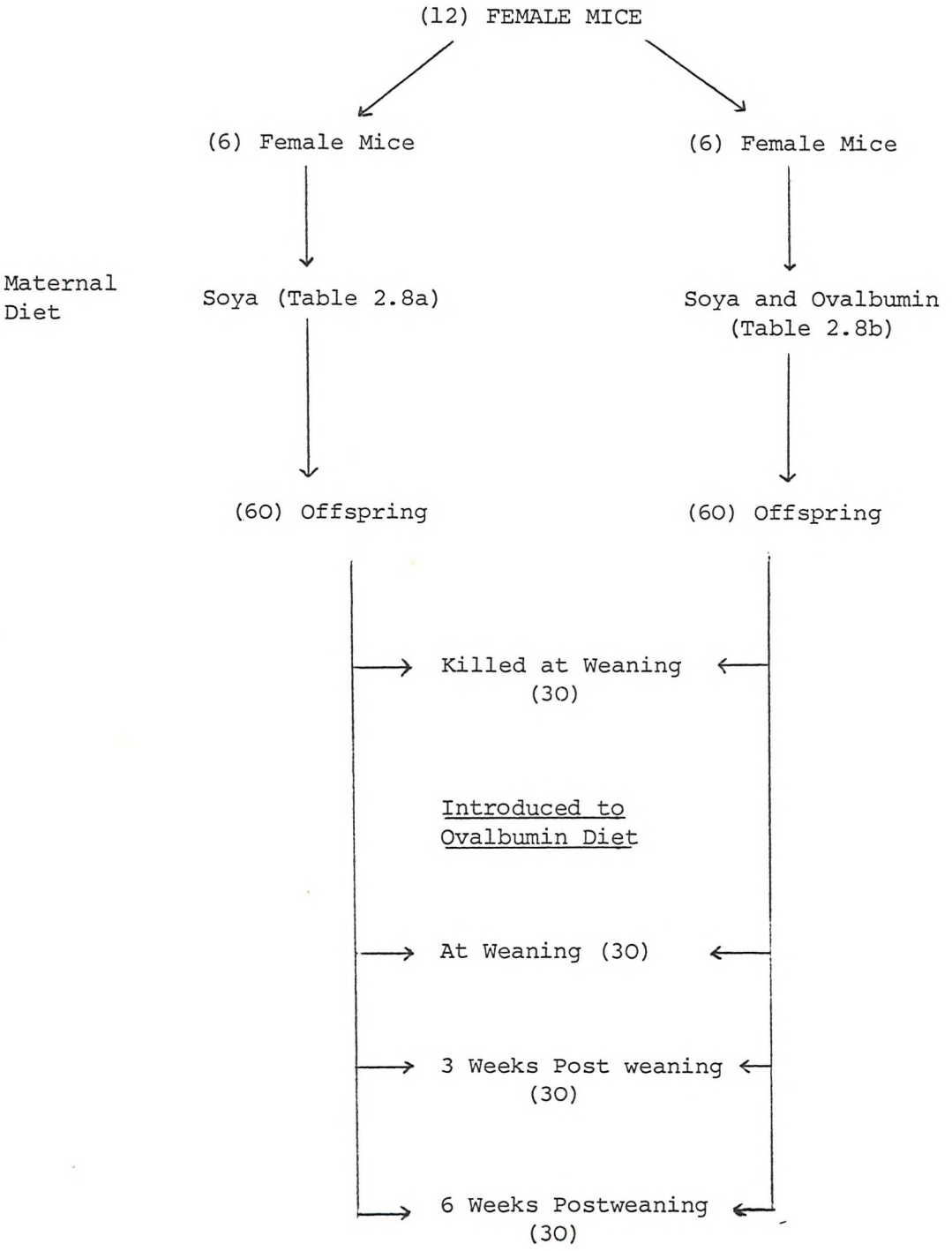
1) At weaning (3 weeks post partum)
2) 3 weeks post weaning
3) 6 weeks post weaning

At each of the above ages, 30 mice were introduced to the diet containing ovalbumin, 15 from Group X and 15 from Group Y. The weanling mice were fed the control soya based diet which did not contain ovalbumin (Table 2.8a) until they reached the correct age for introducing the ovalbumin diet.

A diagramatic representation of the experimental design is presented in Fig. 5.2.

Each group of 30 mice were killed by decapitation 3 weeks after they had been introduced to a diet containing ovalbumin. The serum from each group, including a group which had been killed at weaning, was collected and stored at -22^{o}C. The serum was diluted 1:20 with PBST for the measurement of serum anti-ovalbumin lgG by the ELISA method (Section 2.1.1, Method 1). The concentration of ovalbumin used to coat the was 10 µg/ml and the antibody label employed was Rabbit anti Mouse lgG conjugated to Horse Radish Peroxidase (Nordic Immunochemicals Laboratories). The results were expressed as the optical density observed at 405 nm after the addition of the assay mixture, containing O-phenylene diamine as the chromogen.

FIG. 5.2 EXPERIMENTAL DESIGN



The 30 mice introduced to ovalbumin at different ages were killed after 3 weeks on the ovalbumin diet. The serum from each group and those killed at weaning were measured for antiovalbumin lgG.

Results

The data were analysed by 2-way analysis of variance and the mean values compared, using Duncan Multiple Range Test (Table 5.3). Mean values for serum anti-ovalbumin lgG at weaning were significantly higher in those offspring weaned from mice fed ovalbumin. This is in agreement with the results of experiment 5a (Table 5.1). In mice born of non-ovalbumin fed breeding stock, the age at introduction of ovalbumin into the diet of the offspring had no effect on the capacity to develop circulating lgG to ovalbumin. The capacity to develop circulating antibodies to ovalbumin in mice bred from ovalbumin fed breeding stock varied with age of ovalbumin introduction. Those introduced to ovalbumin at three weeks post weaning show significantly lower circulating anti-ovalbumin lgG, measured three weeks later, than comparable mice introduced at weaning. Similarly, the hyporesponsiveness of the group introduced at 3 weeks appeared to have waned such that those introduced to ovalbumin at 6 weeks post weaning showed a significantly higher response.

TABLE 5.3

Mean Serum Anti Ovalbumin lgG in Mice Introduced to Ovalbumin at Different Ages and Killed Three Weeks after Antigen Introduction

Age at which Weanling Mice were fed Ovalbumin (Weeks Post Weaning)	Group X Maternal Breeding Stock not Exposed to Ovalbumin		Group Y Maternal Breeding Stock Exposed to Ovalbumin
0	0.53 ± 0.017[a]	*	1.093 ± 0.06[abc]
0-3	1.66 ± 0.084[b]	*	1.172 ± 0.085[ac]
3-6	1.223 ± 0.134[c]	*	0.809 ± 0.087[b]
6-9	1.4 ± 0.146[c]	NS	1.236 ± 0.116[c]

* - Significant difference in serum antiovalbumin lgG between the two groups at a given time ($P < 0.05$)

a-c - Mean values within a given dietary group without a common superscript differ significantly ($P < 0.05$)

NS - No significant difference in serum anti ovalbumin lgG between the two groups at a given time

Discussion

Mice bred from mothers which had not been exposed to ovalbumin (Group X) were able to produce specific serum antibodies when challenged with ovalbumin at any age from weaning to 6 weeks post weaning. The antibody response was greater in those animals introduced to ovalbumin at weaning, compared with those introduced at 3 and 6 weeks post weaning. This ability to develop serum antibodies at any age from weaning to maturity is similarly observed in the rabbit (Experiment 3b).

The mice born from maternal stock which had been exposed to ovalbumin (Group Y) confirmed the observation made in experiments 5a and 5b, that the weanling mice had significant levels of specific serum antibodies. Serum antibodies were also observed in the mice challenged with ovalbumin at o, 3 and 6 weeks post weaning.

The presence of maternal antibody in the serum of the offspring may mask any unresponsiveness exhibited by the neonatal immune system. This possibility has been discussed in experiments 5a and 5b. However, it is unlikely that the antibodies in mice that have ingested ovalbumin from 6-9 weeks post weaning is maternally derived. This can be inferred from experiment 5b (Table 5.2), which shows a significant decline in the circulating anti-ovalbumin lgG in mice bred from ovalbumin fed dams in which the progeny were maintained on an ovalbumin free diet. It is reasonable to assume that the lower levels of anti-ovalbumin lgG in this group observed at 3 weeks post weaning would have declined further by 6 weeks post weaning. This group, with the minimal retention of maternal antibodies were able to mount an immune response to dietary protein comparable to animals from non-ovalbumin fed breeding stock.

Other studies in mice have demonstrated a specific suppressed immune response in the offspring from mothers who have been injected with antigen during pregnanay *(Halsey & Benjamin, 1976)*. Experiments in which the litters have been cross-fostered do not agree as to the route by which the tolerance is transferred *(Auerbach & Clark, 1975; Yamaguchi et al., 1983)*. Whereas *Hanson et al. (1979)* showed that oral tolerance to ovalbumin was abolished by a prior parenteral injection

which instead resulted in a secondary antibody response. A similar observation was made by *Strobel et al. (1981)*, who found that ingestion of a specific dietary protein soon after birth did not lead to immunological tolerance when later challenged with the antigen, but primed the animal for a greater immune response.

In this study, preweanling exposure to ovalbumin either in utero, via the maternal milk, or by nibbling the food in the maternal cage, may have primed the animal to produce serum antibodies when re-exposed to ovalbumin at some stage post weaning.

CHAPTER 6

THE IMMUNOLOGICAL RESPONSE PRODUCED BY THE ACUTE INGESTION OF COWS MILK (A COMMON DIETARY PROTEIN AND GOATS MILK (A NOVEL DIETARY PROTEIN) IN MAN

Introduction

Although the human infant receives passive immunity in the form of maternal lgG in utero and therefore has some protection against possible pathogens at birth, the newborn infant is still particularly vulnerable to antigen challenge.

Grusky & Cooke (1955) and *Roberton et al. (1982)* have shown that the gut of the premature and newborn infant absorbs greater quantities of protein in a macromolecular form than the adult or older child. This may be due to structural immaturity of the neonatal gut *(Moxey & Trier, 1975)*, or the lack of secretory lgA.

During the neonatal period there appears to be a lack of secretory lgA within the intestine of the neonate which has also been found to be devoid of antibody producing cells in the lamina propria *(Selner et al., 1968)*. Secretory lgA is the predominant immunoglobulin present in intestinal secretions and in the mucus coating the intestinal absorbtive cells of the gut *(Tomasi et al., 1965)* and it appears that this class of immunoglobulin protects the intestinal epethelium from antigenic penetration *(Walker, 1975c)*. In slgA deficiency, macromolecules including partially digested proteins and bacterial breakdown products, are absorbed across the gut in quantities which are capable of evoking a systemic immune response resulting in an increased incidence of gastrointestinal disease *(Hong & Ammann, 1972)*.

The natural diet during the neonatal period is human colostrum and breast milk, which have been shown to possess a wide variety of host resistant factors which include cellular components such as T and B lymphocytes, macrophages and other mononuclear cells *(Diaz-Jouanen & Williams, 1974)*. Breast milk also contains immunoglobulins of which lgA is probably the most important *(McClelland, 1982)*. Cells which actively synthesize and secrete lgA are present in the milk and their development has been found to be closely related to antigenic stimulation of the gastrointestinal tract *(Goldblum et al., 1975)*. This close relationship between antigenic stimulation of the gastrointestinal tract and the lgA produced in the mammary gland is thought to be due to the selective transfer of sensitized lymphocytes from the gut to the mammary gland *(McClelland, 1982)*. Since the mother is exposed to the microorganisms and other antigenic macromolecules which the infant is likely to encounter, the passive immunity provided by

this mechanism is believed to be important in protecting the secretory IgA deficient gut of the newborn infant from absorbtion of pathogenic quantities of antigenic material.

The quantity of IgA in the colostrum and mature milk in relation to the total protein content is illustrated in Table 6.1, which shows that 54% of the total protein in colostrum is IgA and 14% of the total protein in mature milk is IgA.

Ogra et al. (1977) have studied the absorbtion of IgA from the colostrum by the neonate and they found that only 10 - 15% of ingested IgA appeared in the serum of the infant. The majority of IgA ingested (60%) was excreted by the faeces. The remaining 25 - 30% was believed to reflect loss due to either proteolytic degradation or retention and the intestinal mucosal surfaces. The ability of sIgA to resist digestion appears to be due to the secretory component *(Steward, 1971)*. The majority of the ingested sIgA by the infant from the colostrum remains in the intestine and has been described as a 'proteolytic-resistant antiseptic paint', which functions to protect against pathogenic invasion of the newborn gut mucosa *(Heremans et al., 1966)*. However, when an infant's nutritional requirements must be met by foods other than the mothers milk, a foreign protein will ordinarily be fed and the immunological as well as nutritional consequences of a particular food and feeding regime should be considered. Estimates of the incidence of cows milk allergy vary from 0.1 - 8.0% of the population *(Freier & Kletter, 1970)* and it is more prevalent among infants that have been bottle rather than breast fed from birth *(Buisseret, 1978)*. A study of the immunological consequences of feeding infants with cows milk and soy products concluded that when the mothers milk is not obtained, the preferred substitute would be a heat treated, lower protein, cows milk based product, rather than a soya product *(May et al., 1982)*. Feeding a soya product from birth did not have any advantage over feeding cows milk as the antibody response to pasteurized cows milk introduced after initial soya feeding was significantly greater than after cows milk products had been fed initially. Other studies have confirmed that soy protein is at least as antigenic as milk protein and does not appear to have any prophylactic effect against atopic diseases *(Kjellman & Johansson 1979, Easthman et al., 1978)*.

The immunological consequences of infant feeding practices may not

only be important in the prevention of atopic diseases such as bronchial asthma, allergic rhinitus, atopic dermatitis, allergic uticeria and certain gastrointestinal disorders, but also the raised serum antibodies produced after the ingestion of an antigenic foreign protein may result in the formation and eventual deposition of circulating immune complexes. Circulating immune complexes have been reported in a wide variety of diseases, including systemic lupus erythematosus, various forms of glomerulo-nephritis, rheumatoid artheritis and chronic inflammatory bowel disease *(W.H.O. Scientific Group, 1977)*. Immune complexes, especially those composed of antibodies against cows milk have been implicated in the aetiology of atherosclerosis *(Poston, 1979)*.

The following experiment was designed to study the effect of acute ingestion of a novel protein on the immune system of the adult. Goats milk was chosen as the protein to be used in the study as it is not consumed regularly or in large quantities by the majority of the population. Furthermore, it was also readily available and considered fairly palatable by most people.

Novel proteins are increasingly being introduced into the modern diet. Soya protein is a good example; although soya is frequently consumed the immunological consequences of consuming large quantities of this protein are still being investigated. Goats milk is often used as a cows milk substitute, either in infant feeds or by individuals who cannot consume cows milk *(Taitz & Armatage, 1984)*.

Specific serum antibodies, antigen and total immune complexes were measured after consuming goats milk and cows milk.

TABLE 6.1

Average Composition of Protein in Human Colostrum and Mature Human Milk

	Colostrum		Milk	
	Amount g/l	% of Total Protein	Amount g/l	% of Total Protein
Total Protein	100	100	11	100
Casein	(16)	(16)	4.5	41
Lactalbumin	(11)	(11)	3.1	28
Lactoferrin	14	14	1.5	14
Serum Albumin	1.6	1.6	0.3	2
Lysozyme	0.4	0.4	0.1	1
Immunoglobulin A	54	54	1.5	14

Figures in brackets are estimated amounst

(Department of Health & Social Security, 1980)

Materials and Methods

Subjects

Thirteen volunteers, 8 male and 5 females, were recruited from the postgraduate population and teaching staff at the Southampton University Medical School.

None of the subjects had a previous history of allergy or gastro-intestinal disease, although one subject reported a certain amount of intolerance to lactose. None of the subjects had previously consumed goats milk although some had consumed goats milk products (feta cheese, yoghurt) but only rarely and not in the 6 months prior to the experiment.

Milk Products

Pasteurized cows milk: Unigate Dairies, Aldershot, Hants.
Goats milk: Whitcher's Dairy, Starrs Green Lane, Battle, East Sussex.

Experimental Design

The 13 subjects were divided into two groups of seven and six. Each group was exposed to an acute ingestion of cows milk and goats milk on separate occasions.

The experimental sessions commenced at 9.0 - 9.30 a.m. and prior to this each subject was expected to have fasted for the preceding 12 hours. At the beginning of each experiment a butterfly cannula was introduced to a superficial vein of the lower forearm of every subject and a 10 ml blood sample was taken. The cannula was then flushed with a solution of heparin (200 units in 1 ml) to prevent the blood from clotting in and around the needle. When subsequent blood samples were required the first 1 ml taken was discarded to prevent heparin contamination.

The first experiment consisted of the subjects consuming as much goats milk as possible over a 20 minute period, either with or without the addition of milk flavourings. The volume the subjects consumed ranged between 2 - 3 pints which is 37.8 - 56.7g protein. After the subjects had finished drinking the time was recorded and a 10 ml blood sample was taken after 30 minutes, subsequent 10 ml blood samples were taken after 60, 90, 120 and 180 minutes. After each blood sample was taken, the needle was flushed with the heparin solution. The blood was allowed to clot for 1 hour at room temperature, then the clot was

was contracted by incubating for 1 hour at 4°C. The serum was collected by centrifugation and aliquoted into 0.5 ml samples and stored in epindorph vials at -70°C. The subjects were asked to return after two weeks when the second experimental session was performed, in which the equivalent quantity of cows milk was consumed.

Analytic Methods

Circulating Specific lgG Antibodies

Antigoats milk protein lgG antibodies and anti-cows milk protein lgG antibodies were monitered by ELISA (2.1.1). A concentration of 30 µg/ml in carbonate buffer of dried skimmed milk (St. Ivel Ltd., Trowbridge, Wiltshire) or a volume containing 30 µg protein per ml of skimmed goats milk (Whitchers Dairy, Starrs Green Lane, Battle, East Sussex) was used to coat the cuvettes. The skimmed goats milk was prepared by centrifuging whole goats milk at 30,000g for 30 minutes and removing the fat on the surface with a spatula.

The conjugate used to label the circulating antibody was swine antihuman lgG attached to the enzyme label Horse Radish Peroxidase (purchased from Nordic Immunochemical Laboratories). O-Phenylenediamine was used as the chromogen and the results expressed as the optical density recorded at 405 nm.

Circulating Specific Antigen

Circulating antigenic whole cows milk protein, whole goats milk protein and goat immunoglobulin G were measured in the serum by the ELISA system adapted for detecting the presence of specific antigenic protein in the circulation, which has been described in Section 2.3. The cuvettes were coated with 10 µg/ml of antibodies of the lgG class raised against cows milk protein, goats milk protein or Goat lgG. The serum was diluted 1:10 in PBST and added to the cuvettes and the antigen label employed was the enzyme Horse Radish Peroxidase conjugated to either rabbit anti whole cows milk protein lgG, Rabbit anti whole Goats milk protein lgG or Rabbit anti Goat lgG. The conjugates were all prepared by the method given in Section 2.3.2. Standard samples containing different concentrations of specific antigen (100 µg/ml to 1 ng/ml) were run simultaneously to the experimental serum to produce a standard curve so that the concentration of specific antigenic protein or polypeptide fragments present in the serum could be calculated. The

antigens used were Dried Skimmed Cows Milk (St. Ivel Ltd.), Skimmed Goat Milk (Whitchers Dairy) and Goat lgG (Sigma Chemicals). The standard curves for antigenic cows milk protein, goats milk protein and Goat lgG are given in Figs. 6.1, 6.2 and 6.3, respectively.

Total Circulation Immune Complexes

Total serum immune complexes were estimated by the Clq binding technique described in Section 2.4, with alkali aggregated human γ globulins used as standards (2.4.4). The results were expressed as % radiolabelled protein precipitated by TCA which represents the total amount of labelled Clq.

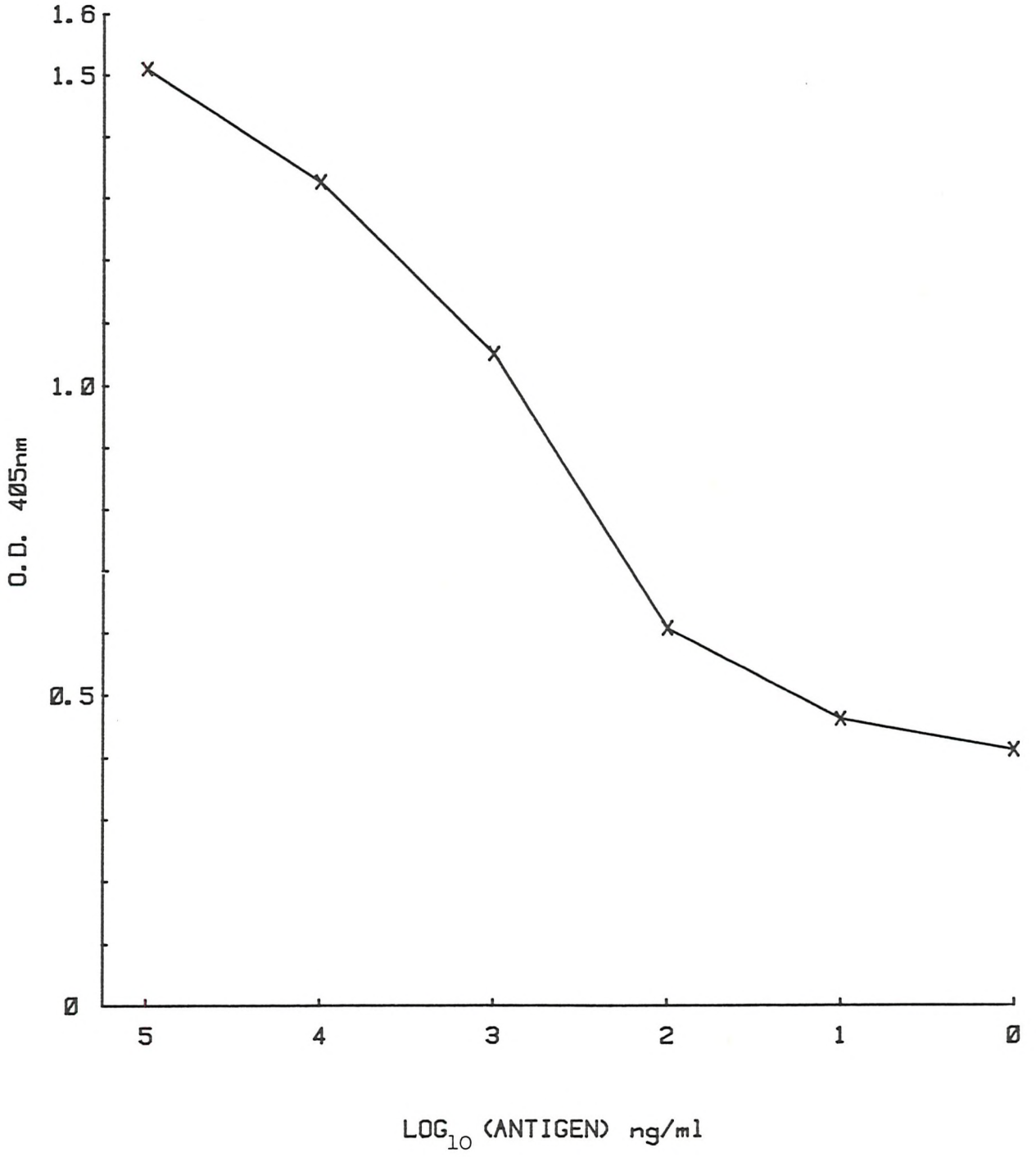


Figure 6.1 STANDARD CURVE RELATING ABSORBANCE AT 405nm TO THE CONCENTRATION OF COWS MILK ANTIGEN (ng/ml).

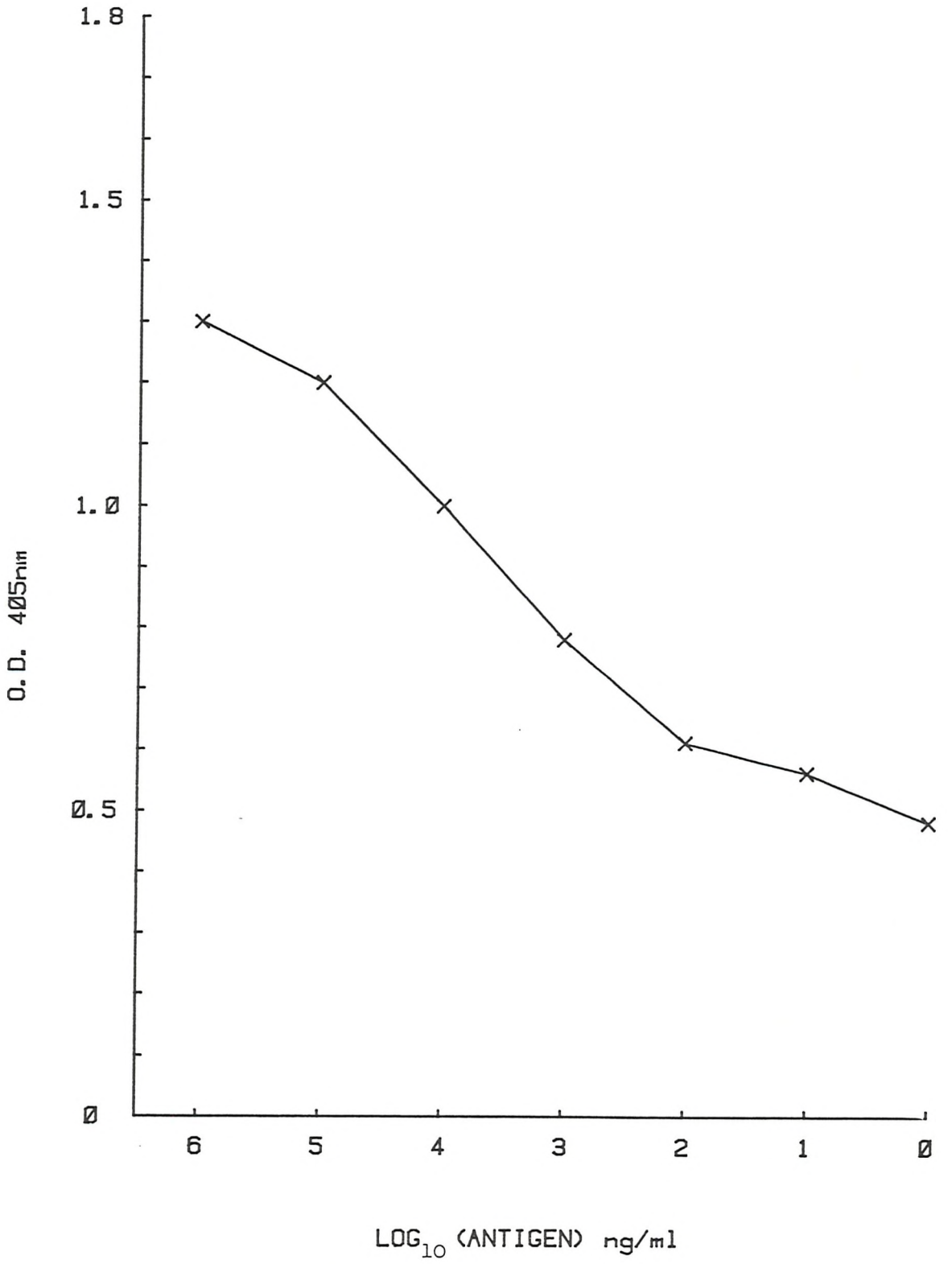


Figure 6.2 STANDARD CURVE RELATING ABSORBANCE AT 405nm TO THE CONCENTRATION OF GOATS MILK ANTIGEN (ng/ml).

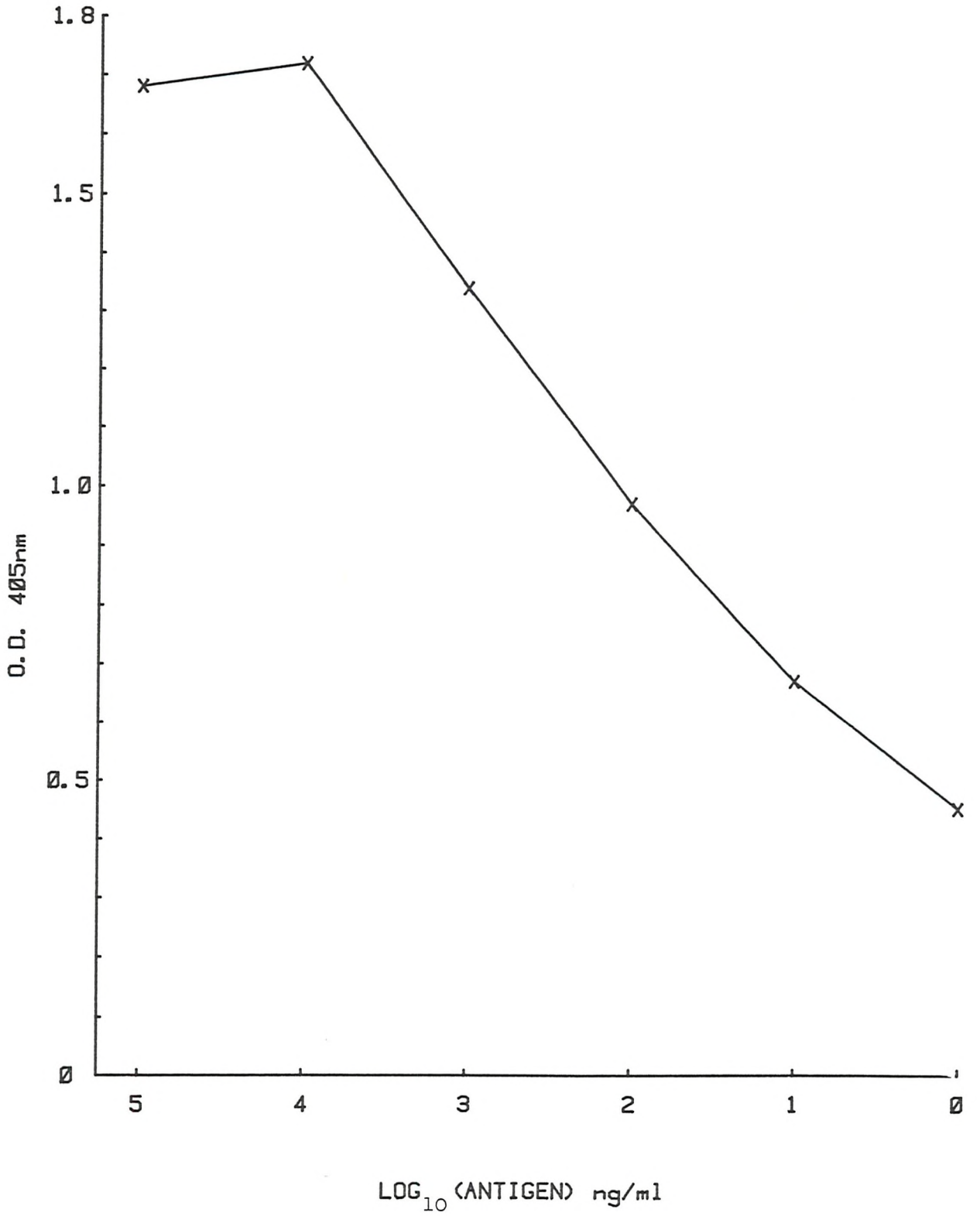


Figure 6.3 STANDARD CURVE RELATING ABSORBANCE AT 405nm TO THE CONCENTRATION OF GOATS MILK IgG (ng/ml).

Results

Specific Serum Antibodies

The level of serum antibodies against whole goats milk and whole cows milk protein was monitored during the experiment and analysis of variance showed that there was no change in antibodies against either of these proteins, following the consumption of the milk. The mean serum concentration of antibodies to cows milk was significantly greater than the mean serum concentration of antibodies to goats milk ($P < 0.001$). The mean concentration of anticows milk protein lgG was found to be significantly greater than the mean concentration of antigoat milk lgG at all times during the experiment. The mean antibody values are given in Table 6.2.

Total Circulating Immune Complexes

The mean total serum immune complex level recorded prior to consuming the milk was 4.56 ± S.D. 1.56. (The high value of subject G was excluded). A positive response was defined as one which exceeded basal levels by 2 standard deviations. Therefore, values greater than 7.7% Clq binding were recorded as positive. The mean serum immune complex results did not increase significantly from baseline levels in the three hours after drinking cows milk or goats milk. There was also no difference in the levels of serum immune complexes after either test meal. The mean total serum immune complex results are presented in Table 6.3, the values are expressed as a percentage of total Clq binding.

Figures 6.4 and 6.5 show the individual immune complex results after drinking cows milk or goats milk. Only subject G produced a significant increase in circulating immune complexes after drinking goats milk.

Serum Specific Antigen

Only two subjects (Subject B and G) had detectable levels of circulating antigenic cows milk protein and goats milk protein after consuming cows milk and goats milk. Both these subjects also had detectable amounts of specific antigenic protein before drinking the milk. The individual serum concentrations of antigenic cows milk protein and antigenic goats milk protein are presented in Table 6.4 and Table 6.5.

Specific serum antigen in Subject B increased from 83 ng/ml to 251 ng/ml 1 hour after drinking cows milk and from 275 ng/ml to 9.549µg/ml

30 minutes after drinking goats milk. Specific serum antigen in Subject G increased from 10 µg/ml to 39.8 µg/ml 30 minutes after consuming cows milk and 100 ng/ml to 2398 ng/ml after consuming goats milk.

Small amounts of antigen were detected in a few other subjects at certain times. The possible presence of antigen in the serum of other subjects must not be excluded as the serum was diluted in PBST 1:10 for the assay, therefore concentrations below 10 ng/ml could not be detected accurately.

The concentration of specific goat immunoglobulin G was also measured (Table 6.6). Circulating antigenic goat IgG was detected in the serum of subjects B and G but in much lower quantities than for whole goats milk or cows milk. Antigenic Goat IgG was also detected in the serum of Subject F 120 minutes after consuming the goats milk.

TABLE 6.2

Mean Concentrations of Specific Serum lgG Antibodies
After Consuming Cows Milk and Goats Milk
(Serum Diluted 1:20)

Time (Minutes after milk consumption)	Diet Consumed: Cows Milk — Concentration of Antibodies against Cows Milk O.D. 405 nm	Diet Consumed: Goats Milk — Concentration of Antibodies against Goats Milk O.D. 405 nm
0	1.930 ± 0.065[a]	1.36 ± 0.098[x]
30	1.928 ± 0.064[a]	1.126 ± 0.100[x]
60	1.89 ± 0.057[a]	1.150 ± 0.097[x]
90	1.906 ± 0.070[a]	1.140 ± 0.123[x]
120	1.866 ± 0.067[a]	1.125 ± 0.101[x]
180	1.89 ± 0.066[a]	1.145 ± 0.105[x]

a,x - Values within a group with a common superscript do not differ significantly

TABLE 6.3

Mean Circulating Immune Complexes (Expressed as % C1q Binding) after Drinking Cows Milk and Goats Milk

Diet	Time (Minutes after drinking the milk)					
	0 (Before Drinking)	30	60	90	120	180
Cows Milk	5.04 ± 0.56	5.38 ± 0.457	4.6 ± 0.403	4.84 ± 0.54	4.94 ± 0.60	4.73 ± 0.55
Goats Milk	4.61 ± 0.60	4.43 ± 0.54	4.25 ± 0.32	5.05 ± 1.06	5.27 ± 1.47	4.51 ± 0.62

No significant difference in mean circulating immune complexes was observed after drinking goats milk compared to cows milk and there was no change in immune complex levels during the experimental period

TABLE 6.4

Detectable Levels of Specific Serum Antigen (ng/ml) after Drinking Cows Milk

ng of Antigenic Cows Milk Protein

Subject	Quantity Protein Consumed (grams)	Time (Minutes after consuming the milk) 0 Before Drinking The Milk	30	60	90	120	180
A	47.2	0	0	0	0	0	0
B	47.2	83	79	251	140	151	39
C	56.7	0	0	0	0	0	0
D	37.8	0	0	0	0	0	0
E	37.8	0	0	0	0	0	0
F	56.7	0	0	0	0	0	0
G	37.2	10000	39810	19054	19950	3019	3981
H	37.2	0	0	0	0	0	0
I	47.2	0	0	0	0	0	0
J	47.2	0	0	0	0	0	0
K	37.8	0	0	0	0	0	0
L	37.8	0	0	0	0	0	0
M	37.8	0	0	0	0	0	0

(Values calculated from the standard curve where O.D. at 405 nm is plotted against concentration of cows milk protein - Fig. 6.1)

TABLE 6.5

Detectable Levels of Specific Serum Antigen (ng/ml) after Drinking Goats Milk

ng of Antigenic Goats Milk Protein

Subject	Quantity Protein Consumed (grams)	Time (Minutes after consuming the milk)				
		0 Before Drinking The Milk	30	60	90	120	180
A	47.2	0	0	0	0	0	0
B	47.2	275	9549	1584	158	457	151
C	56.7	0	0	0	0	0	0
D	37.8	15.8	10.2	0	0	0	0
E	37.8	0	0	0	0	0	0
F	56.7	0	0	0	0	0	0
G	37.8	100	758	150	10	2398	15.1
H	37.8	0	0	0	0	0	0
I	47.2	0	0	0	0	0	0
J	47.2	0	0	0	0	0	0
K	37.8	0	0	0	0	0	0
L	37.8	0	0	0	0	0	0
M	37.8	0	0	0	0	0	0

Values calculated from the standard curve of O.D. at 405 nm produced by different concentrations of goats milk lgG (Fig. 6.2)

TABLE 6.6

Detectable Levels of Goat lgG in the Serum (ng/ml) after Drinking Goats Milk

ng of Antigenic Goat Immunoglobulin G after consuming Goats Milk

Subject	Quantity Protein Consumed (grams)	Time (Minutes after consuming the milk) 0 Before Drinking The Milk	30	60	90	120	180
A	47.2	0	0	0	0	0	0
B	47.2	15.8	16.8	15	15	10	0
C	56.7	0	0	0	0	0	0
D	37.8	0	0	0	0	0	0
E	37.8	0	0	0	0	0	0
F	56.7	0	0	0	0	0	0
G	37.8	19	26.3	63	15.4	20	27
H	37.8	0	0	0	0	0	0
I	47.2	0	0	0	0	0	0
J	47.2	0	0	0	0	0	0
K	37.8	0	0	0	0	0	0
L	37.8	0	0	0	0	0	0
M	37.8	0	0	0	0	0	0

Values calculated from the standard curve of O.D. at 405 nm produced by different concentrations of Goat lgG (Fig. 6.3)

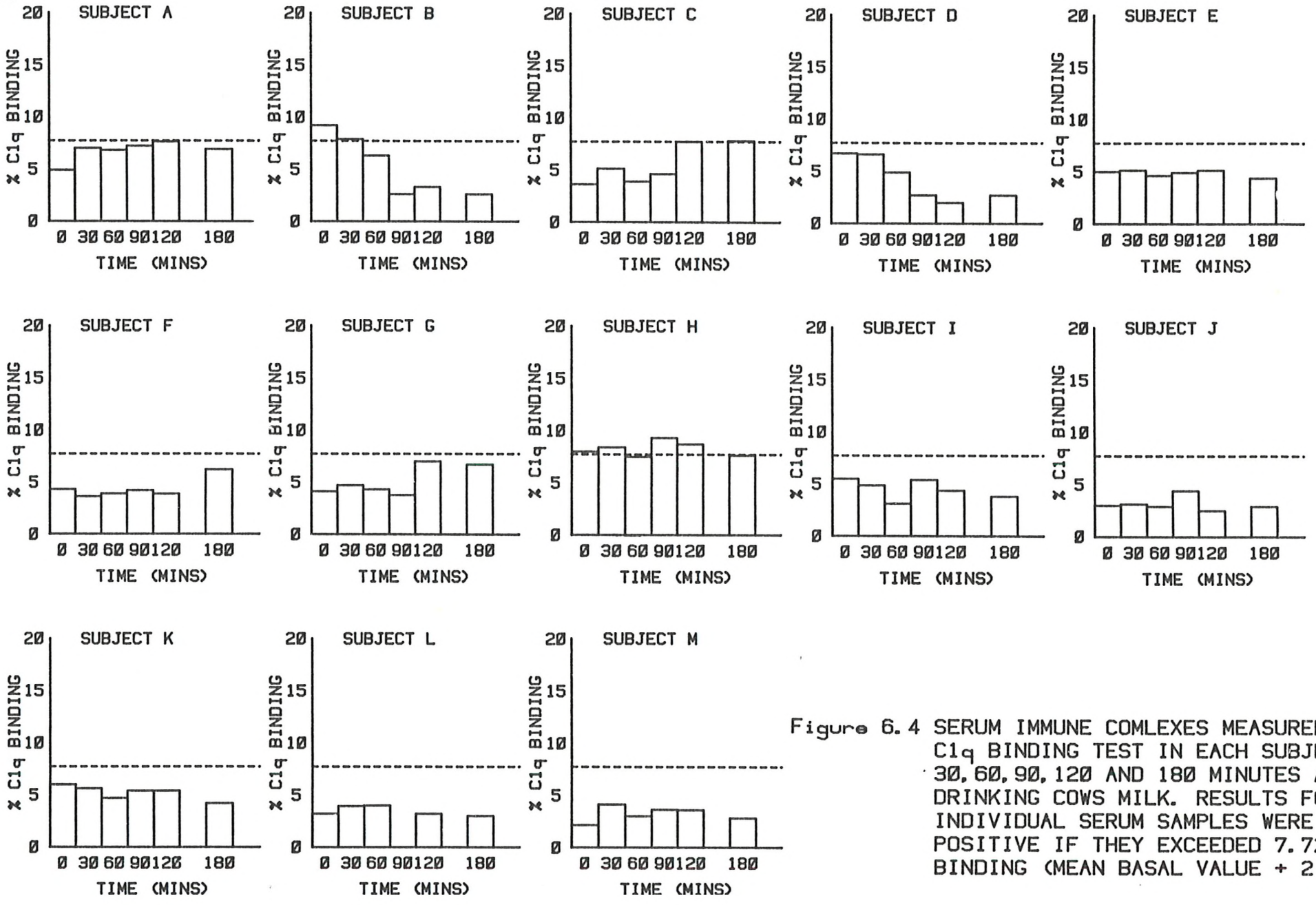


Figure 6.4 SERUM IMMUNE COMLEXES MEASURED BY THE C1q BINDING TEST IN EACH SUBJECT AT 0, 30, 60, 90, 120 AND 180 MINUTES AFTER DRINKING COWS MILK. RESULTS FOR INDIVIDUAL SERUM SAMPLES WERE CONSIDERED POSITIVE IF THEY EXCEEDED 7.7% C1q BINDING (MEAN BASAL VALUE + 2 X SD).

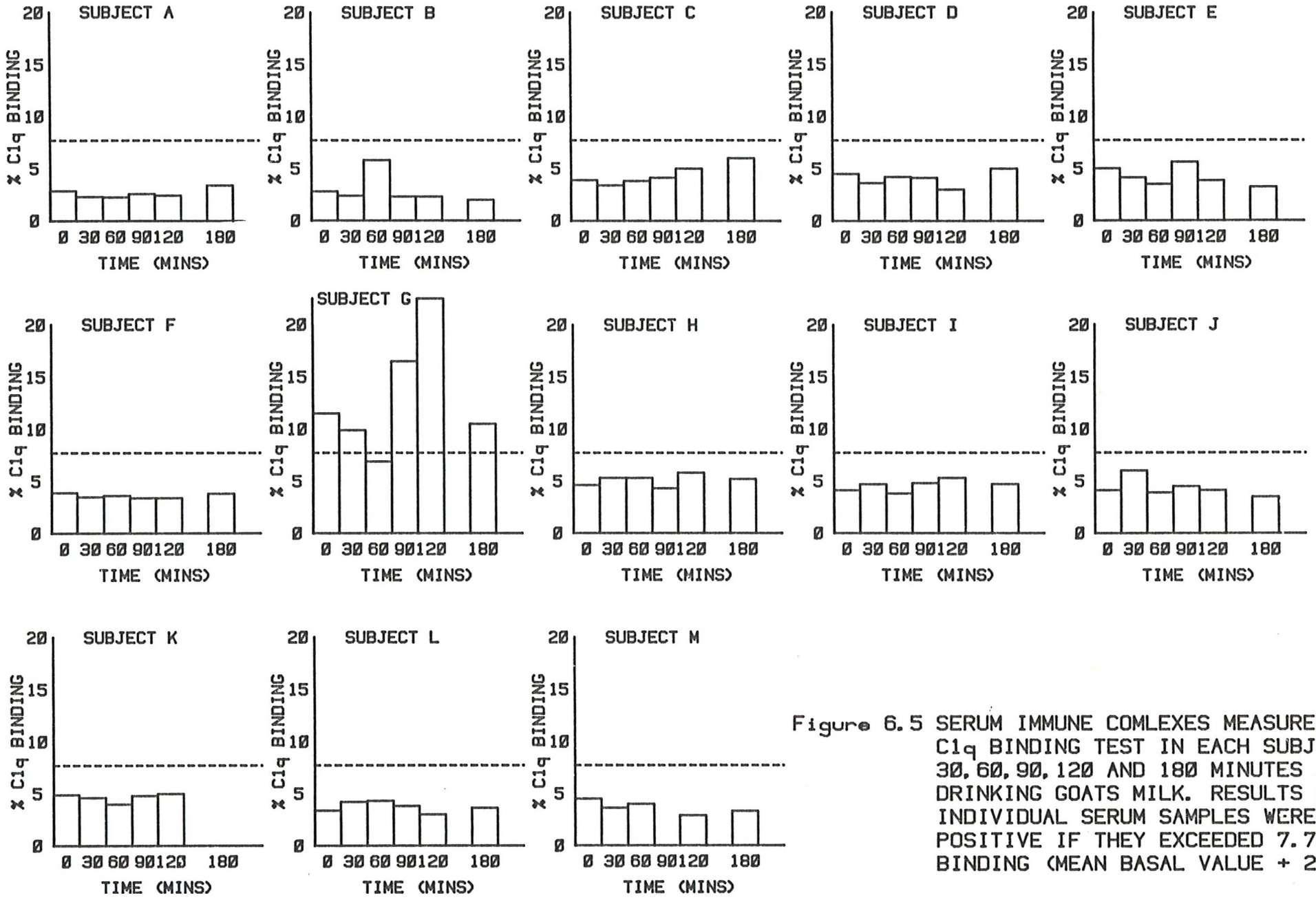


Figure 6.5 SERUM IMMUNE COMLEXES MEASURED BY THE C1q BINDING TEST IN EACH SUBJECT AT 0, 30, 60, 90, 120 AND 180 MINUTES AFTER DRINKING GOATS MILK. RESULTS FOR INDIVIDUAL SERUM SAMPLES WERE CONSIDERED POSITIVE IF THEY EXCEEDED 7.7% C1q BINDING (MEAN BASAL VALUE + 2 X SD).

Discussion

The ingestion of 2 - 3 pints of goats milk or cows milk (37.8 - 56.7 g protein) did not produce any measurable immune response in the majority of volunteers in the following 3 hour period. The lack of serum immune complexes may have been due to an insufficient quantity of specific circulating antibody, although serum antibodies to cows milk protein were detected in all the volunteers. Prior to the experiment, some of the volunteers reported to have consumed goats milk products but then only in small quantities and on rare occasions, while others claimed never to have consumed goats milk products. None of the subjects could recall consuming goats milk products at any time during the preceeding 6 months. Therefore the presence of circulating lgG antibodies to goats milk protein may reflect the ability of antibodies to cows milk protein to bind goat milk proteins. Cross reactivity between goat and bovine β lactoglobulin has been reported *(Freier & Kletter, 1970)*.

A second explanation for the absence of serum immune complexes after the ingestion of the milk is that insufficient antigenic protein was absorbed across the gastrointestinal tract. This explanation is supported by the inability to detect circulating specific antigen in the sera of the majority of subjects after consuming either cows milk or goats milk. Bovine dairy products form a substantial portion of the Westernized diet and this continued exposure to bovine milk proteins may have induced an efficient intestinal antigen specific secretory lgA system by stimulation of the GALT, resulting in immune exclusion *(Walker et al., 1972A)*. Secretory lgA is able to complex with specific antigen preventing its absorbtion into the epethelial cell by pinocytosis. The formation of membrane bound immune complexes have also been found to be more rapidly degraded by local proteases than free antigen *(Walker et al., 1975B)*. The importance of slgA in preventing antigen absorbtion in the adult intestine is substantiated by the detection of significant levels of immune complexes after the ingestion of 100 mls of cows milk (3.33 g protein) in the serum of patients with slgA deficiency. Patients with a more severe lgA deficiency had serum immune complex levels which exceeded the levels in patients with only partial lgA deficiency and circulating immune complexes were not detected in the control subjects after consuming the milk *(Cunningham-Rundles et al., 1979)*. A study by *Pagenelli et al. (1979)* reports a transient rise in circulating immune

* However only dimeric IgA is removed by the hepato-biliary system and as man has very little dimeric IgA in the circulation an alternative system must be present

complexes in normal volunteers following the ingestion of 1.2l litres of cows milk (40.3 g protein) which was exaggerated in the atopic subject. However, *Levinsky et al. (1981)* have shown that normal non-atopic subjects did not produce a rise in circulating Clq binding immune complexes after the ingestion of 1.2l litres of cows milk, but they did develop IgA complexes within 30 minutes after ingesting the milk. These returned to baseline levels after 90 minutes. They suggest that any antigen that reaches the circulation in the non-atopic individual is rapidly removed by combination with monomeric IgA. The fact that monomeric IgA does not activate complement by the classical pathway or elicit other damaging reactions makes it an ideal immunoglobulin for safely eliminating food proteins. In contrast to the IgA complexes formed in response to food challenge in non-atopic individuals, the atopic subjects were observed to form Clq binding immune complexes which appeared as two peaks.

In this study, one subject (Subject G) did produce a substantial rise in Clq binding complexes which peaked at 120 minutes after consuming goats milk. Only a small increase in circulating immune complexes was detected after consuming cows milk in this subject. The measurement of specific serum antigen after ingestion of milk illustrated that a significant quantity of antigen was reaching the circulation in Subject G. A small rise in serum antigen was observed 30 minutes after consuming goats milk with the maximum concentration being observed after 20 minutes. Following the ingestion of cows milk a greater concentration of antigen was observed in the circulation which was maximal 30 minutes after drinking the milk. It may be relevant that at the start of this experiment, this subject did report symptoms of cows milk allergy after the ingestion of large amounts of cows milk. Only one other subject (Subject B) had detectable quantities of specific antigen present in the serum after consuming goats milk or cows milk, the greatest concentration of antigen was observed in the circulation 30 minutes after consuming goats milk. Both subjects (B and G) had antigen present in the serum before consuming both milks. The antigen detected prior to consuming goats milk is likely to be cows milk antigen, again indicating a certain amount of cross reactivity between cows milk and goats milk protein. The immune complex results were positive in Subject B before consuming cows milk and positive in Subject G before drinking goats milk.

The presence of small quantities of antigen in the serum of other subjects were only detected occasionally, however, quantities of antigen in amounts that could not be detected by the assay may have been present. *Levinsky et al. (1981)* did find that small quantities of antigen are absorbed into the serum of normal individuals, but the concentration obtained was not greater than 3 ng/ml after consuming 1.21 litres cows milk (40.3 g protein). A two peak distribution of serum antigen was observed. The first peak occurred at 30 minutes after consuming the milk and the second after 4 - 5 hours. This may represent two different routes of antigen absorbtion, the first into the portal circulation and the second into the lymphatics. A greater quantity of antigen was observed in the atopic individual after consuming the same quantity of milk (15 ng/ml).

These results suggest that the normal healthy adult is able to cope with the ingestion of quantities of milk which have a total protein content of approximately 37.8 - 56.7 g. This is possibly due to an efficient mucosal immune system. The quantity of protein that was found in the circulation of Subjects B and G suggests an ineffective immune exclusion mechanism. The immune exclusion mechanism in the intestinal mucosa can possibly only cope with a critical quantity of antigenic material and the amount of protein which saturates this process may differ in each individual. A previous study *(Goulding, 1982)* has shown an increase in serum Clq binding complexes in healthy subjects after the ingestion of 66g of milk or egg protein with peak levels of circulating immune complexes occurring between 30 and 120 minutes after consumption of the test meal. This small increase in ingested protein may cause an increase in macromolecular protein absorbtion in the majority of individuals allowing the formation of circulating immune complexes.

The ingestion of a novel protein such as goats milk protein would have been expected to produce a greater immune response due to the absence of prior stimulation by the GALT, allowing an increased quantity of angigen to reach the circulation. However, the results from this study suggest that goats milk is not treated as a novel protein as antibodies against bovine milk appeared to bind to goats milk protein. The secretory immune system present in the gut stimulated by the ingestion of bovine milk may also cross-react with goats milk protein in the gastrointestinal tract.

CHAPTER 7

THE IMMUNE RESPONSE TO COWS MILK PROTEIN AND THE DEVELOPMENT OF ATHEROSCLEROSIS IN RABBITS

Introduction

Systemic lgG antibodies to heated milk protein have been related to coronary heart disease in a number of investigative *(Davies et al. 1969; Davies et al., 1974)* and epidemiological reports *(Annard, 1967)*.

Mathews et al. (1974) postulated a role for circulating immune complexes in various vascular disorders. Immune complexes containing lgG can stimulate platelet aggregation with the release of vasoactive amines *(Henson & Spiegelberg, 1973)*, leading to increased vascular permeability and therefore predispose to atherosclerosis thrombosis and embolism. This hypothesis is supported by *Füst et al. (1978)* who reported a high incidence of circulating immune complexes in the sera of patients with acute myocardial infarction, compared to healthy controls. However, other studies have reported no elevation of anti-milk antibodies in patients suffering from coronary heart disease *(Toivanen et al., 1975; Scott et al., 1976)* and raised serum immune complexes have been observed in a variety of cardiac disorders *(Gallagher et al., 1982B)*. Thus the importance of immune complexes in the pathogenesis of atherosclerosis remains unclear.

In a rabbit feeding study it was found that a high fat cholesterol free diet based on soya protein resulted in florid arterial disease and elevated levels of circulating antibody to soya protein *(Gallagher et al., 1978)*. This study was developed by exposing half the breeding stock to soya. The offspring of these animals showed systemic tolerance to the diet containing soya bean protein. These hyporesponsive animals showed lower serum antisoya antibodies, fewer positive serum immune complex results and after one year, less aortic atheroma than the equivalent group in which the breeding stock had not been exposed to soya *(Gallagher et al., 1982A)*. As the other variables such as serum lipid levels were similar in the two groups, it appeared that the animals which showed systemic tolerance to soya protein were protected from atherosclerosis. Cross fostering experiments have shown that the tolerance can be acquired by preweanling exposure to the antigen, either in utero or via the maternal milk *(Gibney & Gallagher, 1982)*.

Soya accounts for less than 1% of the protein consumption in Britain, whereas the average consumption of milk protein is more than 20g protein per day *(Gallagher et al., 1983)*. Thus, the following experiment was designed to investigate the immune reactions to dietary milk protein on

the development of atherosclerosis in rabbits. To do this a high energy milk protein diet was fed to rabbits which induces mild hypercholesterolaemia and more aortic disease than a stock laboratory diet *(Goulding, 1982)*.

Experimental Design

Three weanling New Zealand White Rabbits were fed a semi purified milk protein diet (Table 2.5). At six months of age, all the animals were mated and the fourteen offspring made up Group 1 (6 males and 8 females).

Group 2 was composed of 6 male and 5 female weanling New Zealand White Rabbits reared from a breeding colony which had been fed a milk free, fish meal protein diet over many years (Table 2.1). The two groups of animals had similar body weights at the start of the experiment (Group 1, 1.66 kg ± 0.144; Group, 1.53 kg ± 0.064). All the animals were housed in individual stainless steel cages and from weaning they were fed the cows milk semi purified diet (Table 2.5) for one year.

The rabbits were allowed free access to food and water at all times. Food intakes and body weights were recorded every four weeks and blood samples were taken from the marginal ear vein at monthly intervals. The serum was stored in 0.5 ml aliquots at -70^{o}C and awaited analysis.

Two animals from each group died during the course of the experiment and these animals were not included in the results and statistical analysis. None of the deaths could be ascribed to atherosclerosis.

After one year on the experimental diet, the rabbits were killed by an intravenous dose of pentobarbitone (2 ml/kg body weight) and the aorta was removed and examined for atherosclerosis.

Analytic Methods

Serum Analysis

Serum antibodies to whole milk protein and the individual milk proteins were measured by ELISA (Section 2.1.2 Method (ii) - Microtitre plates (Dynatech Ltd., Billinghurst, Sussex) were coated with 60 µg/ml of bovine dried skimmed milk (St. Ivel Ltd., Trowbridge, Wiltshire) or 20 µg/ml of casein, β lactoglobulin, α lactalbumin, or Bovine Serum Albumin (purchased from Sigma Chemicals, Poole, Dorset). Peroxidase conjugated Goat anti Rabbit lgG (Nordic Immunochemicals) was used as the label and O-dianisidine HCl as the chromogen. The optical density results obtained at .450 nm were expressed as a percentage of a standard reference serum obtained from rabbits which had been immunized against the specific antigen (Section 2.2.1(i). Standard curves of the referernce serum containing antibodies against whole milk protein, casein, β lactoglobulin, α lactalbumin and BSA are given in Figures 7.2, 7.3, 7.4,7.5

and 7.6 respectively.

The concentration of circulating antigenic cows milk protein was also determined by an ELISA which is described in Section 2.3. The cuvettes were coated with 10 µg/ml of anti cows milk protein lgG antibodies. The serum was then diluted 1:10 with PBST and added to the cuvettes. The antigen was labelled by the addition of anti cows milk protein lgG, conjugated to Horse Radish Peroxidase enzyme. The conjugate was prepared by the method described in Section 2.3.2(i). Optical density readings were obtained after the addition of the assay solution containing O-phenylene-diamine as the colour reagent. The results were quantified from a standard curve produced from samples containing known quantities of specific antigen, together with rabbit serum which did not contain the antigen. The standard curve is presented in Fig. 7.7 and the results were expressed as ng of specific antigenic protein per ml of serum.

Serum immune complexes were measured by the Clq method (Section 2.4.3) and cholesterol and triglycerides were determined by the autoanalyser method described in Section 2.5.

Aortic Scoring

The degree of atheroma involvement was measured by placing a transparent grid marked into squares (0.15 x 0.15 cm) over the opened flattened aorta, this method of aortic has been described by *Morgan & Adams, 1974.*

The arota was divided into three regions.

1) The Proximal aorta - the area between the aortic valve and ductus arteriosus scar.
2) The thoracic aorta - the area from the scar to the first major abdominal branch.
3) The abdominal area - the remaining area down to the bifurcation.

Squares containing any disease were counted as positive and the total number was expressed as a percentage of the total area.

The resulting score was checked by staining the aortas with Suden IV and rescored.

(A diagramatic representation of the aortic divisions is given in Figure 7.1).

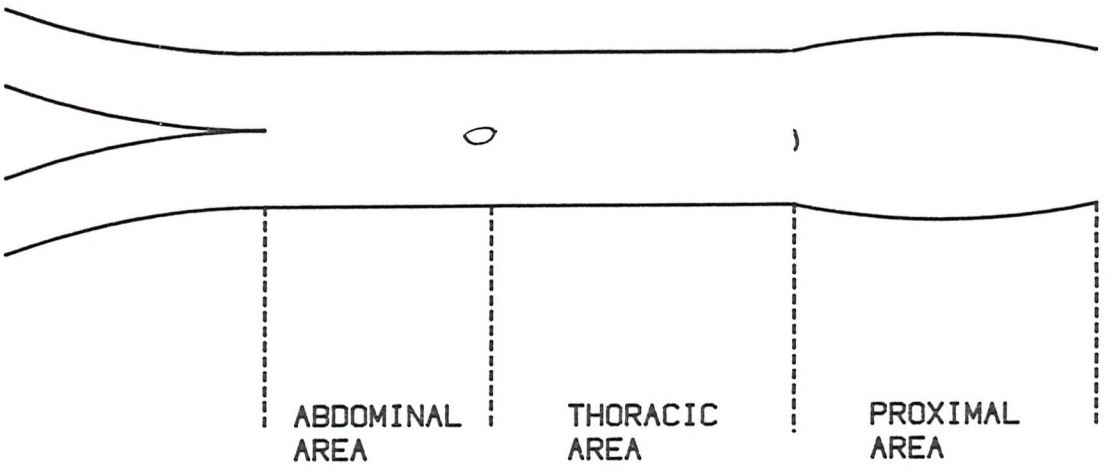


Figure 7.1 THE DIVISIONS OF THE AORTA USED FOR SCORING ATHEROSCLEROSIS.

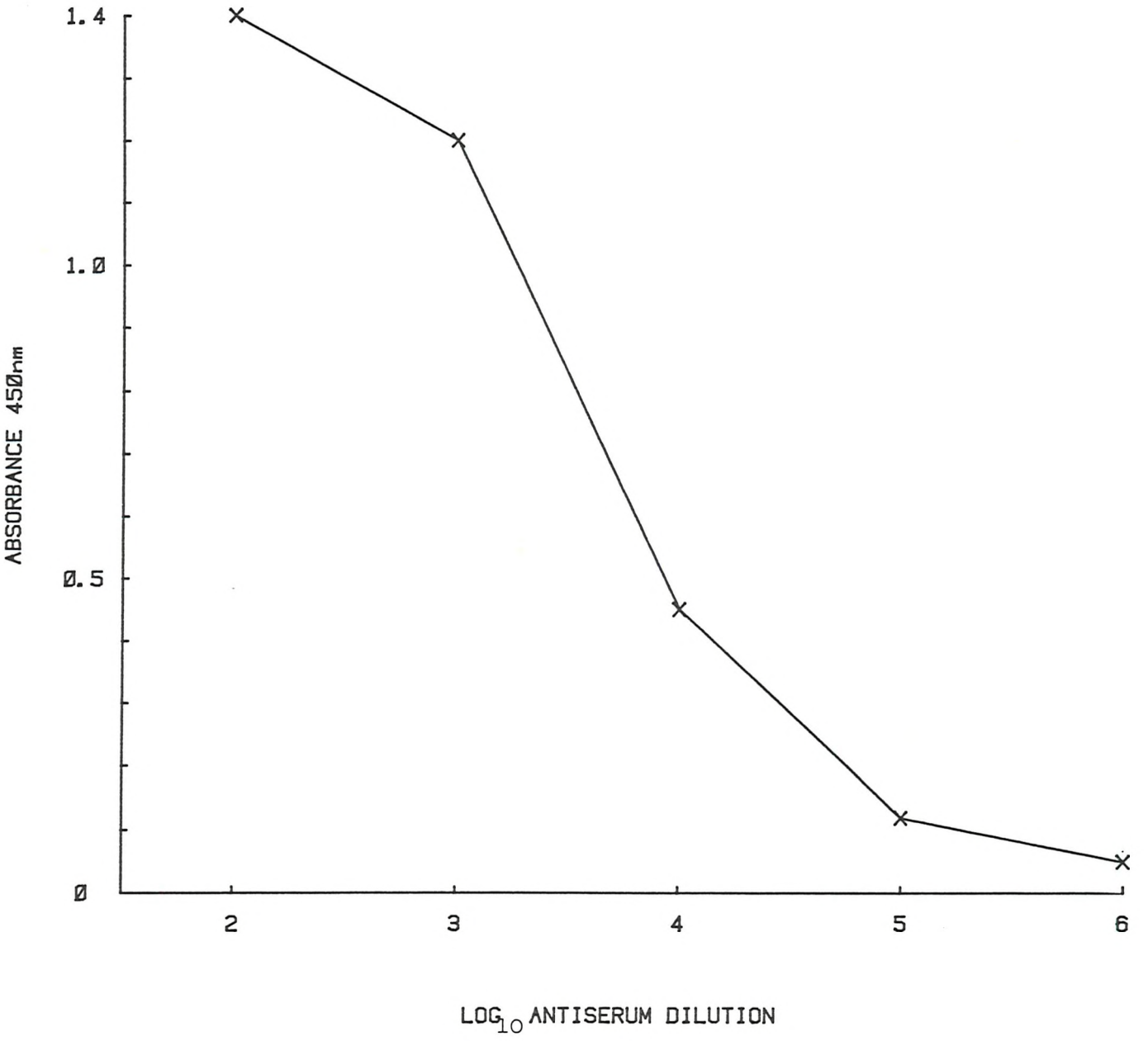


Figure 7.2 STANDARD CURVE. ANTI-MILK PROTEIN IgG MEASURED BY ELISA IN SERUM FROM AN ANIMAL IMMUNIZED AGAINST COWS MILK PROTEIN.

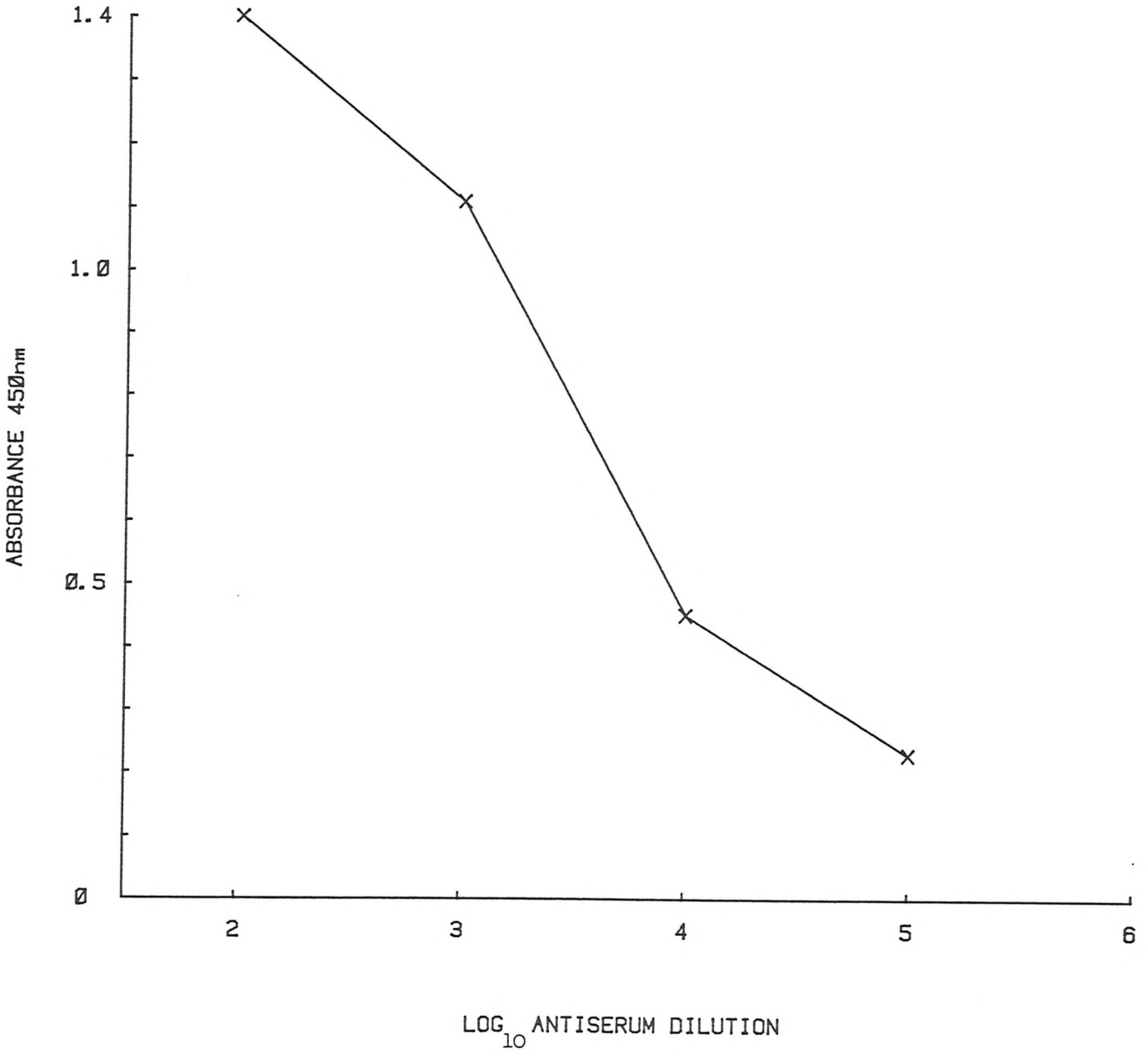


Figure 7.3 STANDARD CURVE. ANTI-CASEIN IgG MEASURED BY ELISA IN SERUM FROM AN ANIMAL IMMUNIZED AGAINST CASEIN.

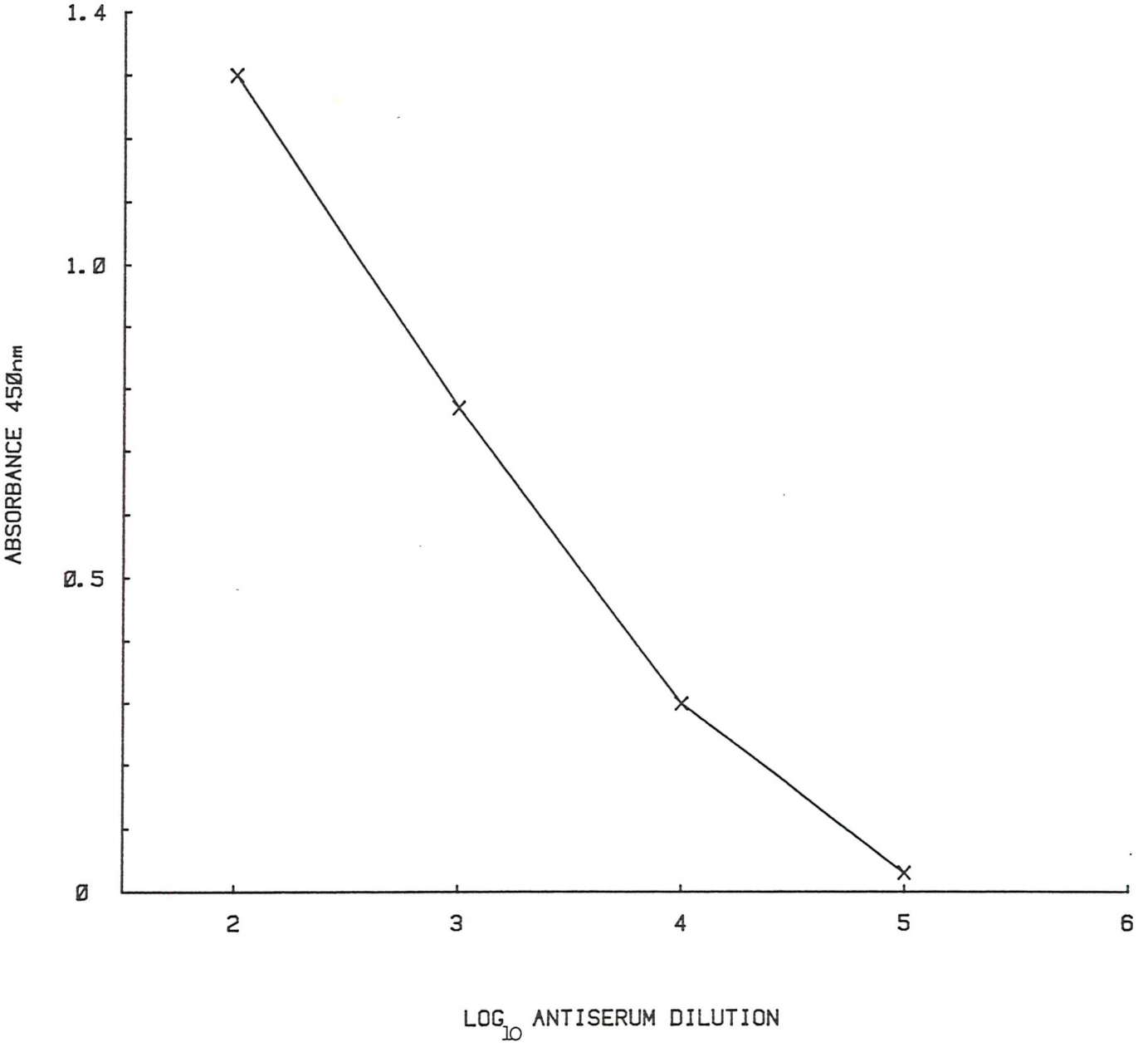


Figure 7.4 STANDARD CURVE. ANTI-βLACTOGLOBULIN IgG MEASURED BY ELISA IN SERUM FROM AN ANIMAL IMMUNIZED AGAINST β LACTOGLOBULIN.

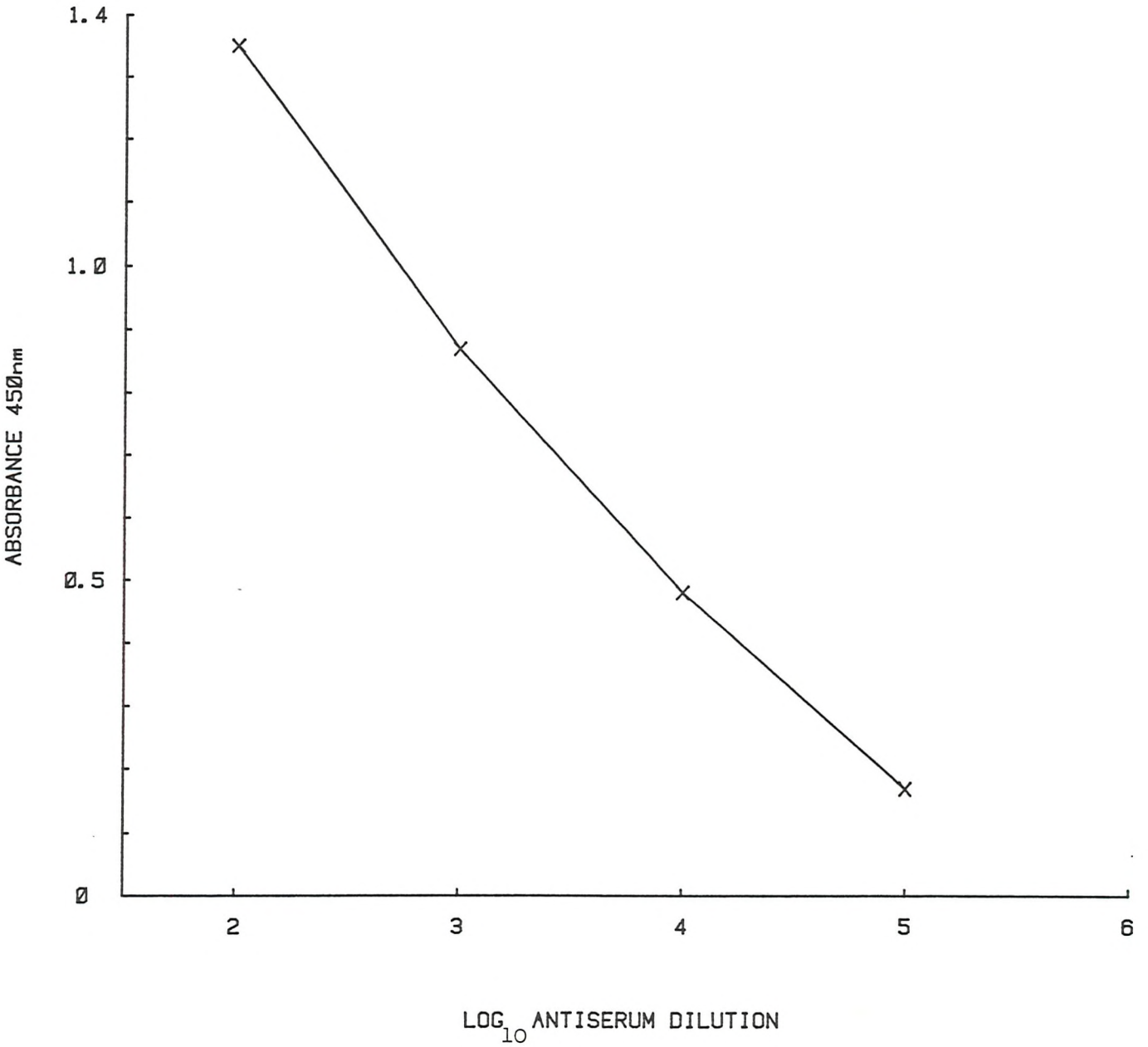


Figure 7.5 STANDARD CURVE. ANTI-αLACTOGLOBULIN IgG MEASURED BY ELISA IN SERUM FROM AN ANIMAL IMMUNIZED AGAINST αLACTOGLOBULIN.

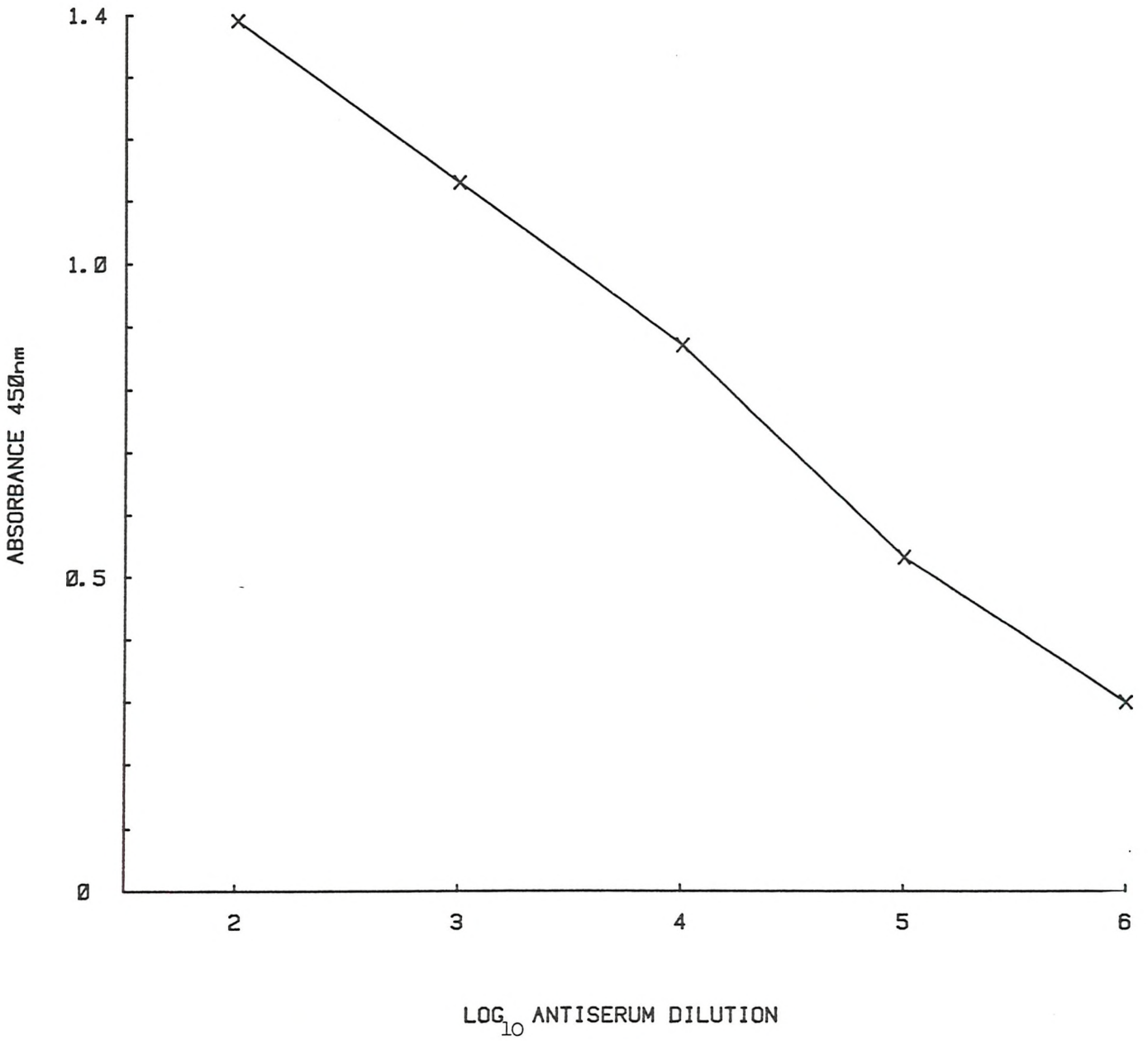


Figure 7.6 STANDARD CURVE. ANTI-BOVINE SERUM ALBUMIN IgG MEASURED BY ELISA IN SERUM FROM AN ANIMAL IMMUNIZED AGAINST BSA.

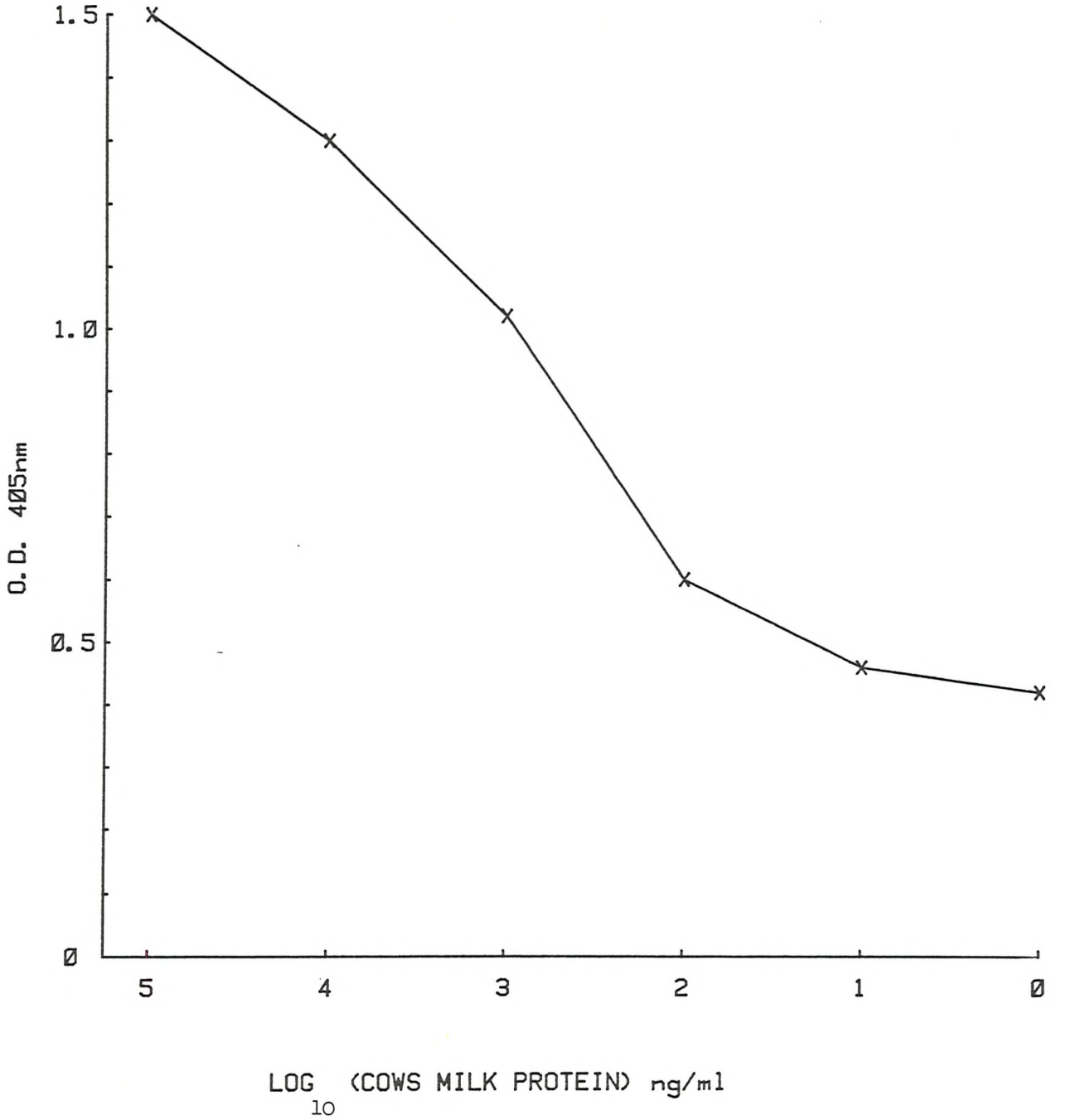


Figure 7.7 STANDARD CURVE RELATING ABSORBANCE AT 405nm TO THE CONCENTRATION OF ANTIGENIC BOVINE MILK PROTEIN (ng/ml).

Results

Live Weight Gains and Food Intakes

The mean body weights of the two groups at weaning were similar and the mean body weights increased steadily over the experimental period. However, one month after weaning and the introduction to the cows milk diet, the group of rabbits from the dams fed cows milk were significantly heavier than the group from dams which had not been exposed to cows milk (Group 1, 2.73 kg ± 0.12(S.E.); Group 2, 2.4 kg ± 0.065 (S.E.)($P < 0.05$)). This difference in the body weights of the two groups observed one month after weaning, was maintained throughout the experimental period (Table 7.1). The increase in the body weights of the two groups during the experimental period is illustrated in Fig. 7.8.

The mean food intakes did not change significantly during the experimental period. Table 7.2 illustrates the difference in food intakes observed at month 1, 6 and 7 ($P < 0.05$). When food intake is expressed per kg body weight the energy intake is similar in the two groups.

Serum Specific Antibodies

The antibody results were expressed as a percentage of a reference serum containing antispecific antibodies. The results were analysed by two way analysis of variants and the mean values were compared using the Duncan Multiple Range Test at the 5% level of significance.

Serum Antibodies to Whole Cows Milk Protein

A significant difference in serum anti lgG against whole cows milk protein was observed between the two groups ($P < 0.001$) and there was a significant increase over the experimental period ($P < 0.001$).

The concentration of anti cows milk protein lgG in the serum of the two groups was not significantly different at weaning and the following 2 months. Three months after the introduction of the cows milk diet, the mean concentration of serum specific antibodies was significantly greater in Group 2 compared with Group 1 and this difference is maintained for the remainder of the experimental period (month 3 to month 12). The mean values are presented in Table 7.3, which illustrates that anti cows milk protein lgG did not increase significantly from weaning in Group 1, except at month 2. In contrast, the mean value for serum

anti cows milk protein lgG in Group 2 increases during the experimental period. The rise in specific serum antibodies during the experimental period is illustrated in Fig. 7.9.

Serum Antibodies Against the Individual Milk Proteins

The serum was analysed for the presence of circulating antibodies to the individual milk proteins at months 0, 4, 8 and 12.

Anti Casein lgG

The concentration of serum lgG specific for casein differed significantly between the two groups ($P < 0.001$) and increased signficantly over the experimental period ($P < 0.001$). The mean values for serum anti casein lgG for the two groups are presented in Table 7.4. The level of specific serum antibodies in Group 1 did not increase significantly from weaning, whereas Group 2 developed increased levels of specific antibodies which were maximal at month 8 (as illustrated in Fig. 7.10), thus resulting in a significant difference in mean serum antibody levels of Group 1 and 2 at months 4, 8 and 12 post weaning.

Anti β Lactoglobulin

Serum anti β lactoglobulin antibodies increased significantly over the experimental period ($P < 0.01$) and a significant increase in serum antibodies occurred with time ($P < 0.001$). The mean values of the two groups are presented in Table 7.5 and illustrated in Fig. 7.11. Mean values of the two groups did not differ significantly at weaning and there is no significant increase in serum specific antibodies in Group 1. Group 2 shows a rise in serum antibodies at 4 months, which is maintained at 8 and 12 months post weaning, resulting in a significant difference in the serum anti β lactoglobulin lgG concentration of the two groups at months 4, 8 and 12 post weaning.

Anti α Lactalbumin lgG

The overall increase in serum anti α lactalbumin lgG was significant ($P < 0.05$) and a significant difference between the two groups also occurred, but again, only at the 5% level. The mean values are presented in Table 7.6, which shows that the mean concentration of specific serum antibodies only differs significantly in Group 1 and 2 at month 8. Serum specific antibodies were not found to increase from weaning in Group 1, whereas the mean value for anti α lactablumin lgG increases in

Group 2 from weaning at month 4 and 8. However, at 12 months, the level of specific antibodies was not significantly different from that observed at weaning (Fig. 7.12).

Anti BSA lgG

Specific serum antibodies increased over the experimental period ($P < 0.01$) and a significant difference was observed between the two groups at the 5% level. However, when the mean values were compared at 0, 4 8 and 12 months by the Duncans Multiple Range Test, there was no difference in the specific serum antibody level in the two groups. The results are presented in Table 7.7. An increase in antibodies occurred in Group 1 at 8 months after the introduction of the milk diet. Group 2 also showed an increase in serum antibodies from weaning at 4 months which was maintained at month 8 and 12 post weaning (Fig. 7.13).

Serum Immune Complex Results

A serum immune complex result was considered positive if it exceeded 7.9% Clq-binding by 7.9% (i.e. mean serum immune complex result from 40 normal rabbits plus two standard deviations).

Fig. 7.14 summarizes the incidence of positive results at months 0, 7 and 10 after the introduction of the cows milk diet. Very few animals had levels of serum immune complexes that were considered positive and there was no difference in serum immune complex formation between the two groups. The mean values for each group are presented in Table 7.8.

Mean Serum Antigen Concentration

A significant difference in circulating antigenic cows milk protein was observed at weaning between the two groups ($P < 0.05$). The mean serum concentration of antigenic cows milk protein at months 0, 4, 8 and 12 after the introduction of the milk diet are presented in Table 7.9. The mean concentration of specific antigen in the serum of Group 1 was not found to change over the experimental period. whereas a decline from the concentration observed at weaning was found in Group 2 after 4 months on the milk diet ($P < 0.05$).

Although the decrease in the concentration of serum specific antigen was not significant from month 4 to month 12 by the Duncan Multiple Range Test (Table 7.9), the linear regression coefficient was similar for both Groups during this period (Fig. 7.15).

Aortic Atheroma

Very little atherosclerosis developed in the rabbits of both groups and the majority of disease that was found was in the proximal region of the aorta. The mean values for the disease in the proximal area were: Group 1, 12.26 ± 5.26% and Group 2, 13.5 ± 6.57% of the total proximal area. The difference between the amount of disease in the proximal area was not found to be significant by the Mann-Whitney U Test. The amount of disease found in each animal is presented in Table 7.10 which illustrates the lack of atherosclerosis in both groups.

Serum Cholesterol and Triglycerides

Serum cholesterol and triglycerides were measured at 3 monthly intervals. The mean serum cholesterol concentrations at weaning were: Group 1, 3.68 ± 0.49 mmol/l and Group 2, 2.94 ± 0.29 mmol/l. Both groups became mildly hypercholesterolaemic during the experimental period. The mean values for each group are presented in Table 7.11, no significant differences in mean serum cholesterol of the two groups developed throughout the experiment.

Total serum triglycerides were also measured at 3 monthly intervals and the mean values are given in Table 7.12. Triglyceride levels were similar in both groups, except at month 12, when the levels in both groups were significantly greater than at other times ($P < 0.05$) and the mean concentration in the serum of Group 1 was significantly greater than Group 2 ($P < 0.05$).

TABLE 7.1

Mean Body Weight (Kg) ± S.E. of Rabbits Fed
A Purified Diet Based on Cows Milk Protein

Maternal Diet	Milk Protein		Fishmeal Protein
Months Post-Weaning	Group 1 (Kg)		Group 2 (Kg)
0	1.66 ± 0.144	NS	1.53 ± 0.064
1	2.73 ± 0.12	*	2.4 ± 0.065
2	3.47 ± 0.105	*	2.98 ± 0.078
3	3.97 ± 0.123	*	3.31 ± 0.146
4	4.10 ± 0.102	*	3.47 ± 0.167
5	4.31 ± 0.096	*	3.64 ± 0.153
6	4.52 ± 0.097	*	3.75 ± 0.169
7	4.67 ± 0.099	*	3.83 ± 0.151
8	4.78 ± 0.09	*	4.0 ± 0.166
9	5.0 ± 0.087	*	4.07 ± 0.156
10	5.10 ± 0.07	*	4.152± 0.157
11	5.30 ± 0.08	*	4.216± 0.167
12	5.5 ± 0.08	*	4.38 ± 0.148

NS - No significant difference between the body weights of the two groups at a particular time.

* - Significant difference between the mean body weights of the two groups ($P < 0.05$) at a particular time

The weight gain in both groups is significant over the experimental period ($P < 0.001$)

TABLE 7.2

Mean Food Intake of Rabbits Fed a Purified Diet Based on Cows Milk Protein (Kg per month)

Maternal Diet	Milk Protein		Fishmeal Protein
Month	Group 1		Group 2
1	2.49 ± 0.133^{a}	*	1.84 ± 0.07^{x}
2	2.50 ± 0.11^{a}	NS	2.35 ± 0.089^{x}
3	2.37 ± 0.13^{a}	NS	2.11 ± 0.117^{x}
4	2.22 ± 0.12^{a}	NS	2.09 ± 0.108^{x}
5	2.3 ± 0.11^{a}	NS	2.07 ± 0.09^{x}
6	2.43 ± 0.13^{a}	*	1.902 ± 0.066^{x}
7	2.51 ± 0.10^{a}	*	1.96 ± 0.114^{x}
8	2.41 ± 0.108^{a}	NS	2.04 ± 0.109^{x}
9	2.28 ± 0.103^{a}	NS	1.99 ± 0.084^{x}
10	2.39 ± 0.076^{a}	NS	2.11 ± 0.076^{x}
11	2.37 ± 0.113^{a}	NS	2.2 ± 0.122^{x}
12	2.29 ± 0.092^{a}	NS	2.07 ± 0.096^{x}

a,x - Values within a given group with the same superscript do not differ significantly

* - Significant difference in the food intake of the two groups at a particular time

NS - No significant difference in the food intake of the two groups at a particular time

TABLE 7.3

Mean Serum Anti Cows Milk Protein IgG after the Introduction of the Milk Based Diet at Weaning (± S.E.)

Age (Months Post Weaning)	Group 1 (Dams Exposed to Milk)			Group 2 (Dams not Exposed to Milk)	
0	0.044	0.04^{a}	NS	0.007	0.005^{a}
1	0.467	0.245^{a}	NS	0.491	0.096^{ab}
2	1.083	0.352^{b}	NS	1.56	0.312^{bc}
3	0.817	0.293^{ab}	*	1.77	0.291^{c}
4	0.623	0.268^{ab}	*	2.72	0.724^{c}
5	0.474	0.197^{ab}	*	2.93	0.868^{c}
6	0.414	0.180^{ab}	*	2.89	0.575^{c}
7	0.796	0.323^{ab}	*	5.07	1.51^{d}
8	0.440	0.163^{ab}	*	7.13	2.00^{d}
9	0.280	0.297^{ab}	*	8.59	1.81^{d}
10	0.880	0.405^{ab}	*	8.84	2.19^{d}
11	0.712	0.346^{ab}	*	7.94	1.77^{d}
12	0.785	0.339^{ab}	*	7.83	1.62^{d}

a,b,c,d - Mean values within a given group without a common superscript differ significantly ($P < 0.05$)

* - Mean values between groups at a given time differ significantly ($P < 0.05$)

NS - No significant difference in the mean values between groups at a given time

Results are expressed as a percentage of reference serum (Fig. 7.2)

TABLE 7.4

Mean Serum Anti Casein lgG after the Introduction of the Cows Milk Diet at Weaning (± S.E.)

Age (Months Post Weaning)	Group 1 (Dams Exposed to Milk)		Group 2 (Dams not Exposed to Milk)
0	0.0 ± 0[a]	NS	0.0 ± 0[a]
4	1.94 ± 1.43[a]	*	4.98 ± 1.86b
8	0.70 ± 0.421[a]	*	9.94 ± 2.78[c]
12	0.576 ± 0.275[a]	*	6.36 ± 1.80[cb]

a,b,c - Mean values within a given group without a common superscript differ significantly ($P < 0.05$)

* - Mean values between groups at a given time differ significantly ($P < 0.05$)

NS - Mean values between groups at a given time do not differ significantly

Results are expressed as a percentage of reference serum (Fig. 7.3)

TABLE 7.5

Mean Serum Anti β Lactoglobulin lgG after the Introduction of the Cows Milk Diet at Weaning (± S.E.)

Age (Months Post Weaning)	Group 1 (Dams Exposed to Milk)		Group 2 (Dams not Exposed to Milk)
0	0.084 ± 0.019[a]	NS	0.007 ± 0.003[a]
4	0.111 ± 0.024[a]	*	5.64 ± 2.21[b]
8	0.071 ± 0.022[a]	*	6.48 ± 2.51[b]
12	0.057 ± 0.013[a]	*	5.82 ± 2.81[b]

a,b,c - Mean values within a given group without a common superscript differ significantly ($P < 0.05$)

* - Mean values between groups at a given time differ significantly ($P < 0.05$)

NS - Mean values between groups at a given time do not differ significantly

Results are expressed as a percentage of reference serum (Fig. 7.4)

TABLE 7.6

Mean Serum Anti α Lactalbumin lgG after the Introduction of the Cows Milk Diet at Weaning (± S.E.)

Age (Months Post Weaning)	Group 1 (Dams Exposed to Milk)		Group 2 (Dams not Exposed to Milk)
0	0.002 ± 0.002^{a}	NS	0.0 ± 0^{a}
4	0.072 ± 0.021^{a}	NS	0.769 ± 0.616^{b}
8	0.067 ± 0.024^{a}	*	2.43 ± 2.19^{b}
12	0.085 ± 0.025^{a}	NS	0.750 ± 0.456^{ab}

a,b,c - Mean values within a given group without a common superscript differ significantly ($P < 0.05$)

* - Mean values between groups at a given time differ significantly ($P < 0.05$)

NS - Mean values between groups at a given time do not differ significantly

Results are expressed as a percentage of reference serum (Fig. 7.5)

TABLE 7.7

Mean Serum Anti BSA lgG after the introduction of the Cows Milk Diet at Weaning (± S.E.)

Age (Months Post Weaning)	Group 1 (Dams Exposed to Milk)		Group 2 (Dams not Exposed to Milk)
0	0.0 ± 0[a]	NS	0.0 ± 0[a]
4	1.63 ± 1.42[ab]	NS	6.38 ± 2.59[b]
8	6.054 ± 2.60[b]	NS	6.04 ± 2.46[b]
12	5.20 ± 2.60[ab]	NS	10.6 ± 3.23[b]

a,b,c - Mean values within a given group without a common superscript differ significantly ($P < 0.05$)

* - Mean values between groups at a given time differ significantly ($P < 0.05$)

NS - Mean values between groups at a given time do not differ significantly

Results are expressed as a percentage of reference serum (Fig. 7.6)

TABLE 7.8

Mean Serum Immune Complex Results expressed as a Percentage of Total Clq Binding

Rabbits Introduced to Cows Milk Diet at Weaning

Age (Months Post Weaning)	0		7		10	
Group 1 (Maternal Exposure to Cows Milk	4.88	0.97	5.35	0.873	3.82	0.46
Group 2 (No Maternal Exposure to Cows Milk	5.73	1.00	6.48	1.20	3.36	0.253

There was no increase in circulating immune complex levels within each group and there was no significant difference in the levels between each group

TABLE 7.9

The Effect of Maternal Exposure to Cows Milk Protein on Serum Concentrations of Antigenic Milk Protein (ng/ml) ± S.E.

Age (Months Post Weaning)	Group 1 (Dams Exposed to Cows Milk)		Group 2 (Dams not Exposed to Cows Milk)
0	99.9 ± 26.45[a]	*	234 ± 50.76[a]
4	105.5 ± 19.14[a]	NS	127 ± 27.83[b]
8	80.9 ± 16.13[a]	NS	117 ± 16.08[b]
12	44.3 ± 9.54[a]	NS	84.1± 18.05[b]

a,b - Mean values within a given group without a common superscript differ significantly ($P < 0.05$)

* - Mean values between the two groups at a given time differ significantly ($P < 0.05$)

NS - Values between the two groups at a given time do not differ significantly

TABLE 7.10

Individual Atherosclerotic Scores. Rabbits Killed after 12 Months on the Experimental Diet

Group 1 - Dams Exposed to Milk Protein

Rabbit Numbers	Proximal Aorta % Disease	Thoracic Aorta % Disease	Abdominal Aorta % Disease
001	14.7	4.0	0
002	14.6	28.4	0
003	44.1	56.5	63.1
005	0	0	0
006	3.2	0	0
007	0	0	0
008	0	0	0
009	4.49	0	0
011	12.1	0	0
012	54.0	0	0
014	0	0	0
018	0	0	0
Mean	12.26 ± 5.26	7.4 ± 5.04	5.25 ± 5.25

Group 2 - Dams Not Exposed to Milk Protein

Rabbit Numbers	Proximal Aorta % Disease	Thoracic Aorta % Disease	Abdominal Aorta % Disease
030	0	0	0
031	9.9	0	0
033	0	0	0
034	0	0	0
035	32.3	0	0
036	54.9	12.3	12.5
037	0	0	0
038	24.5	0	0
041	0	0	0
Mean	13.5 ± 6.57	1.36 ± 1.36	1.38 ± 1.38

TABLE 7.11

Mean Serum Cholesterol Levels (m mol/l) in the Rabbits of Group 1 and Group 2 after the Introduction of the Cows Milk Diet

Age (Months Post Weaning)	Group 1 (Dams Exposed to Cows Milk)		Group 2 (Dams not Exposed to Cows Milk)
0	3.68 ± 0.49^{a}	NS	2.94 ± 0.29^{a}
3	6.04 ± 0.71^{b}	NS	4.72 ± 0.72^{ab}
6	4.21 ± 0.35^{ab}	NS	4.20 ± 0.68^{ab}
9	4.90 ± 0.80^{ab}	NS	5.23 ± 0.78^{b}
12	4.32 ± 0.72^{ab}	NS	4.03 ± 0.48^{ab}

a,b - Mean values within a given group without a common superscript differ significantly ($P < 0.05$)

NS - Mean values between groups at a given time do not differ significantly

TABLE 7.12

Mean Serum Triglycerides (m mol/l) in the Rabbits of Group 1 and Group 2 after the Introduction of the Cows Milk Diet

Age (Months Post Weaning)	Group 1 (Dams Exposed to Milk)		Group 2 (Dams not Exposed to Milk)
0	0.95 ± 0.06^{a}	NS	0.63 ± 0.09^{a}
3	2.10 ± 1.13^{a}	NS	0.82 ± 0.24^{a}
6	1.60 ± 0.52^{a}	NS	0.70 ± 0.17^{a}
9	1.41 ± 0.36^{a}	NS	0.61 ± 0.13^{a}
12	5.61 ± 0.74^{b}	*	3.87 ± 0.58^{b}

a,b - Mean values within a given group without a common superscript differ significantly ($P < 0.05$)

* - Mean values between groups at a given time differ significantly ($P < 0.05$)

NS - Mean values between groups at a given time do not differ significantly

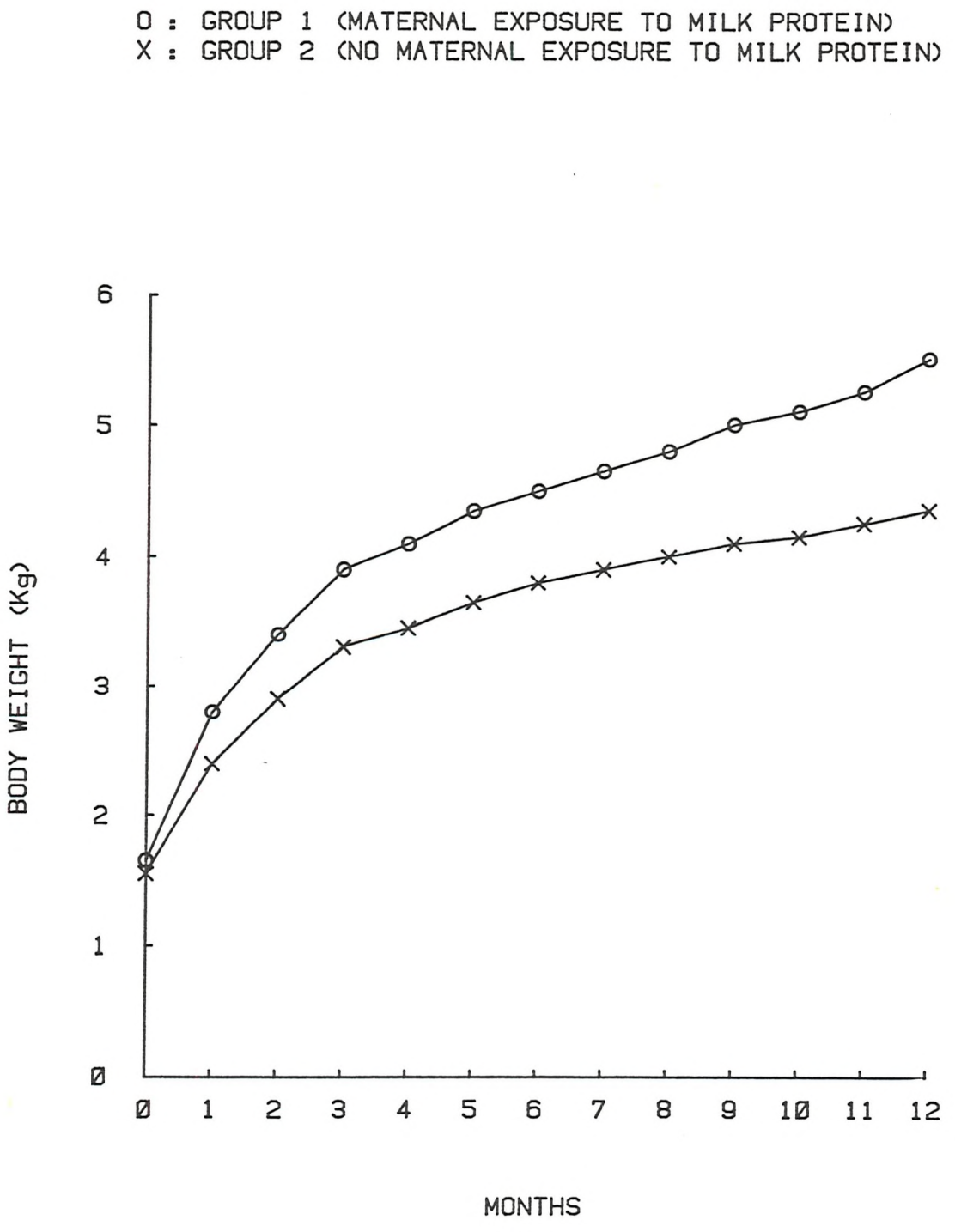


Figure 7.8 INCREASE IN MEAN BODY WEIGHT OF GROUP 1 AND GROUP 2 DURING THE EXPERIMENTAL PERIOD.

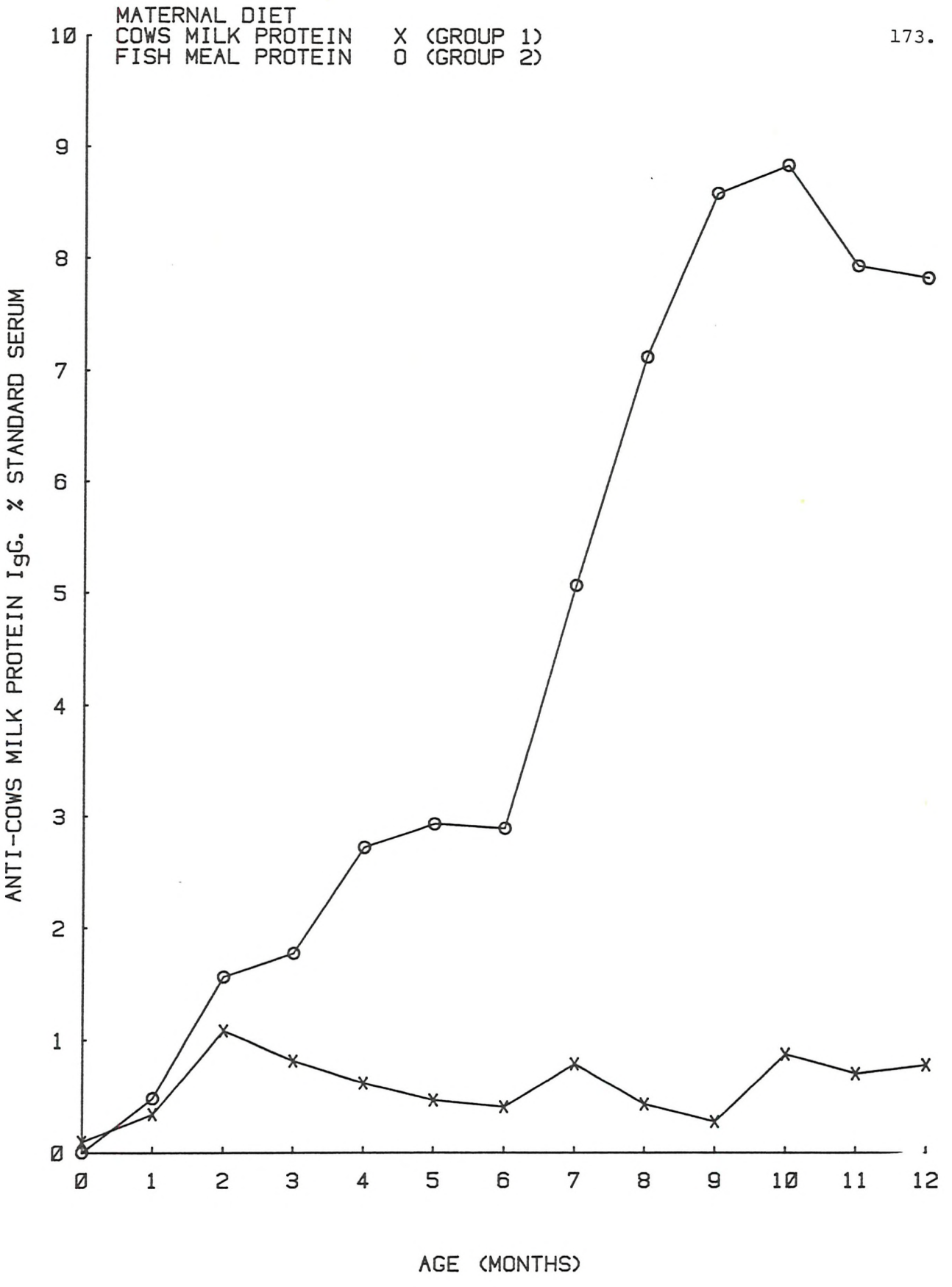


Figure 7.9 THE EFFECT OF MATERNAL EXPOSURE TO MILK PROTEIN ON SERUM ANTI-COWS MILK PROTEIN IgG (% STANDARD SERUM SAMPLE Fig. 7.2) IN RABBITS FED A PURIFIED DIET BASED ON COWS MILK.

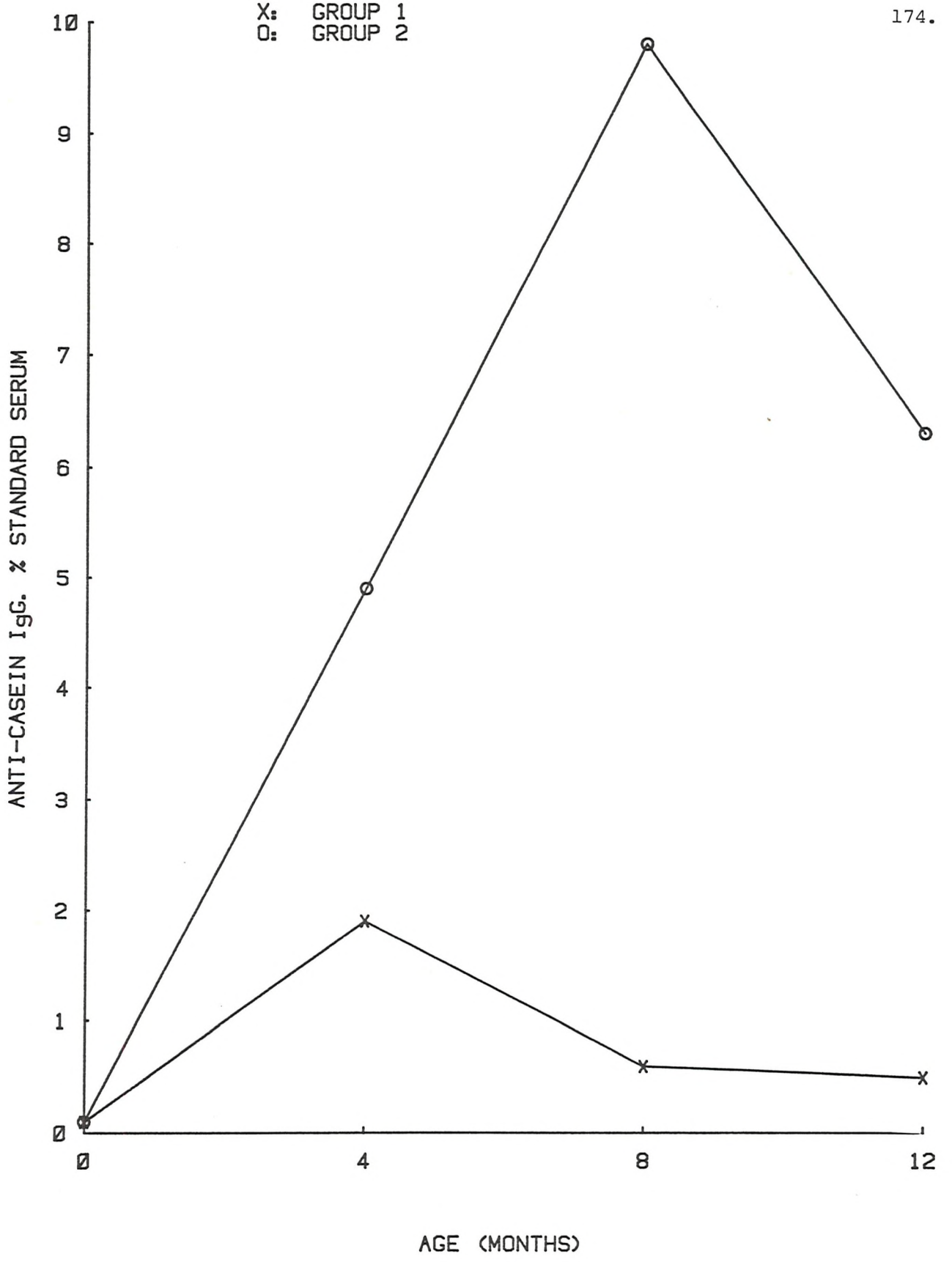


Figure 7.10 THE EFFECT OF MATERNAL EXPOSURE TO MILK PROTEIN ON SERUM ANTI-CASEIN IgG (% STANDARD SERUM SAMPLE Fig. 7.3) IN RABBITS FED A PURIFIED DIET BASED ON COWS MILK.

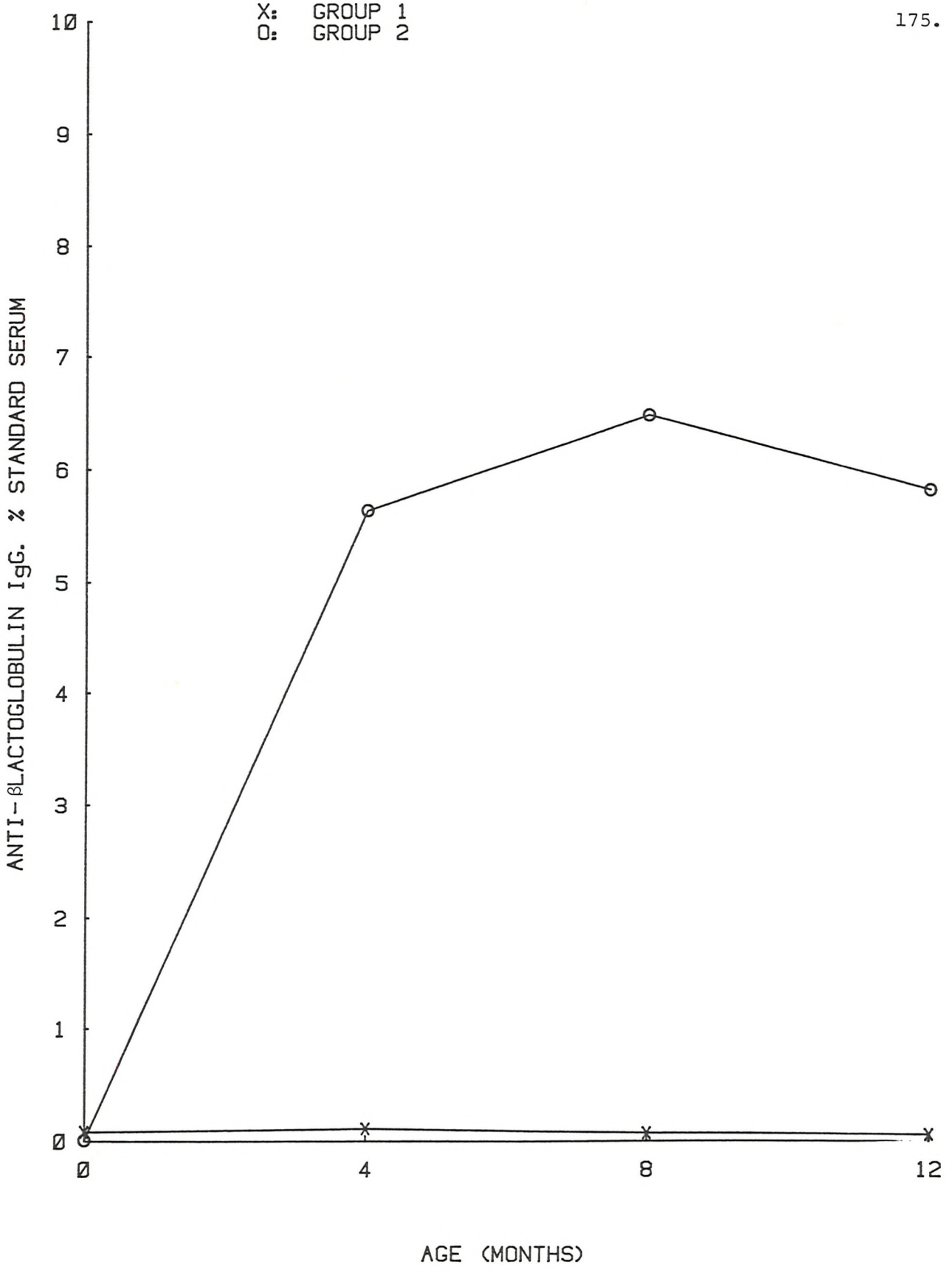


Figure 7.11 THE EFFECT OF MATERNAL EXPOSURE TO MILK PROTEIN ON SERUM ANTI-βLACTOGLOBULIN IgG (% STANDARD SERUM SAMPLE Fig. 7.3) IN RABBITS FED A PURIFIED DIET BASED ON COWS MILK.

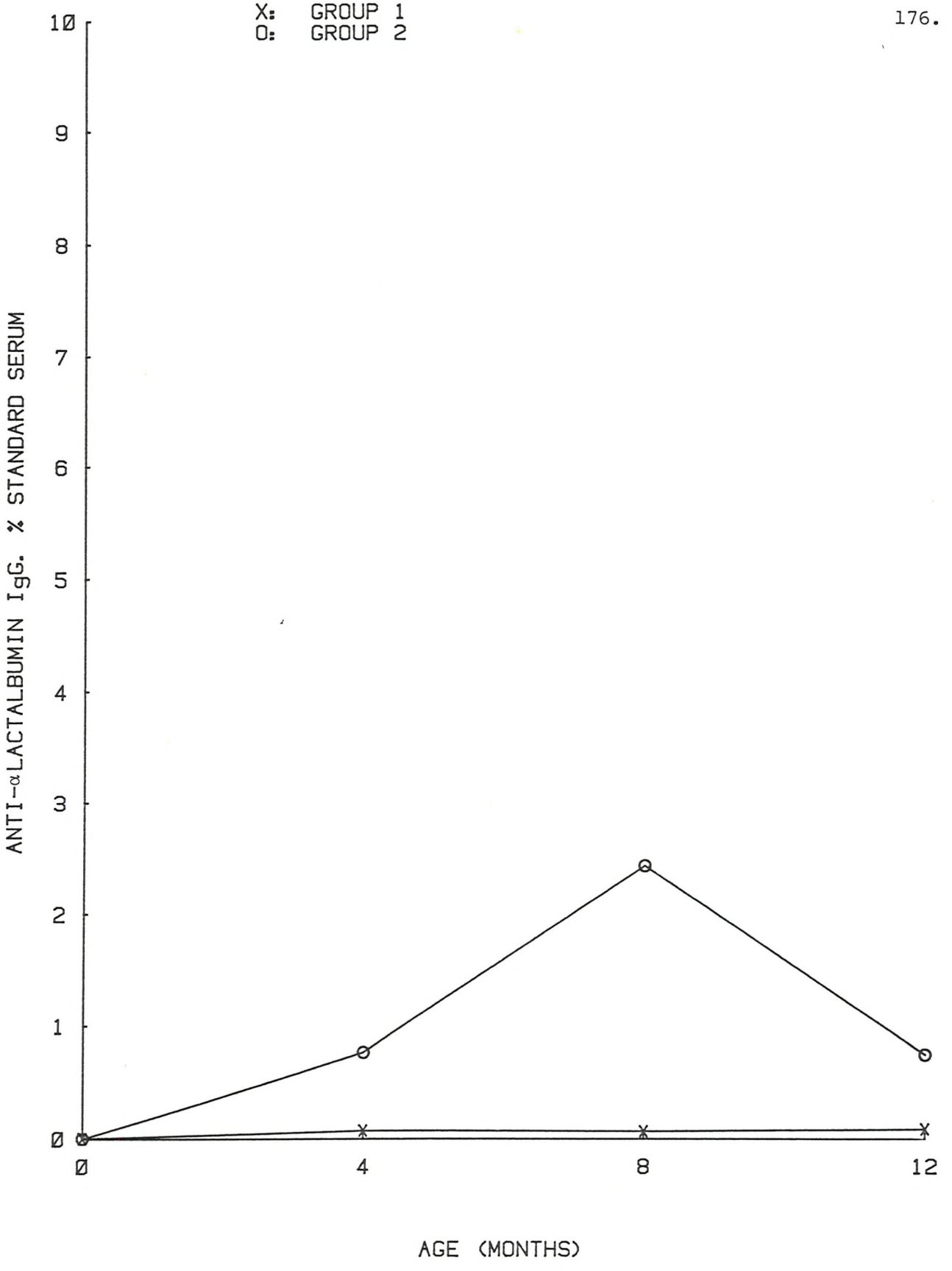


Figure 7.12 THE EFFECT OF MATERNAL EXPOSURE TO MILK PROTEIN ON SERUM ANTI-αLACTALBUMIN IgG (% STANDARD SERUM SAMPLE Fig. 7.5) IN RABBITS FED A PURIFIED DIET BASED ON COWS MILK.

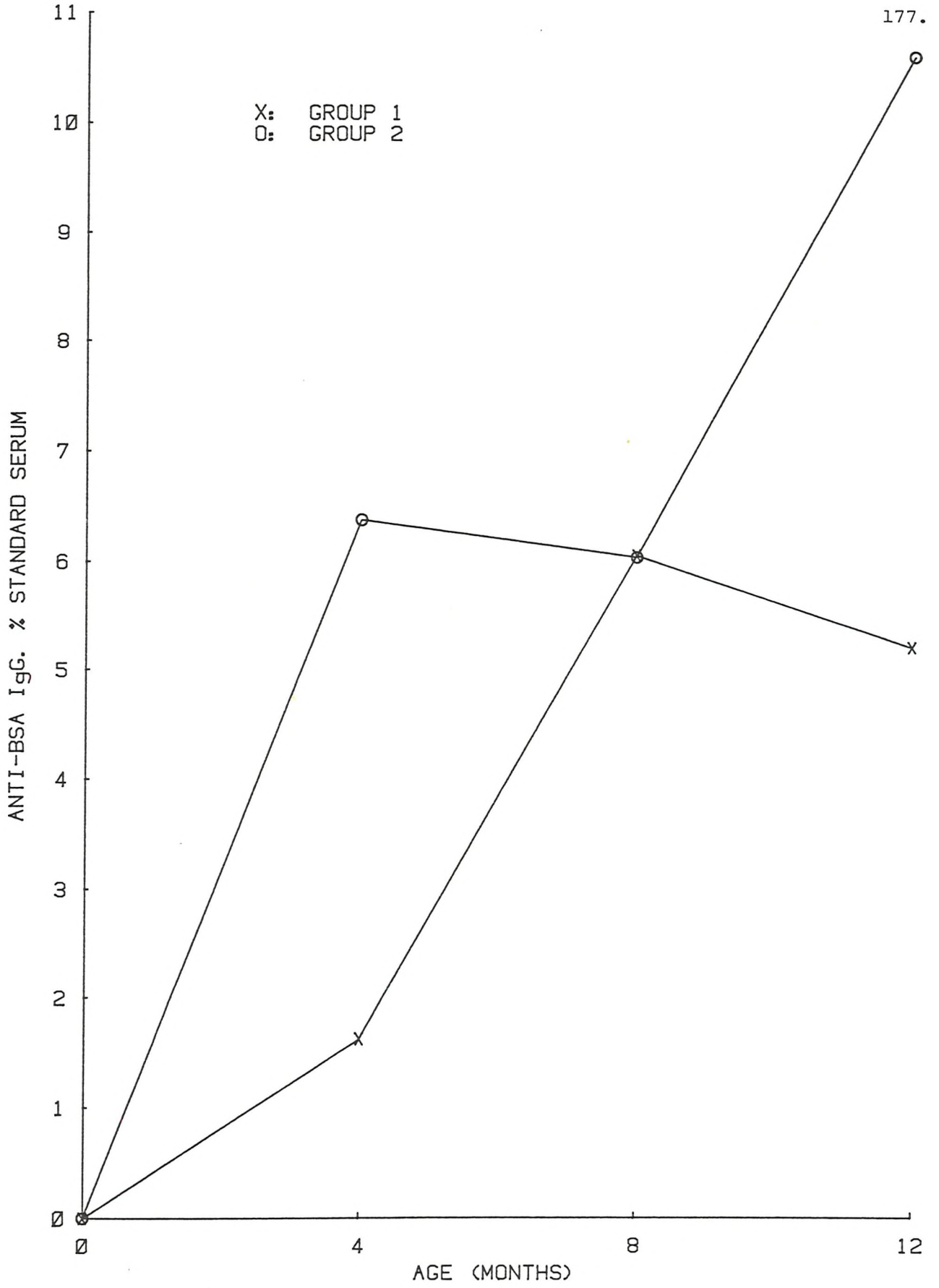


Figure 7.13 THE EFFECT OF MATERNAL EXPOSURE TO MILK PROTEIN ON SERUM ANTI-BSA IgG (% STANDARD SERUM SAMPLE Fig. 7.6) IN RABBITS FED A PURIFIED DIET BASED ON COWS MILK.

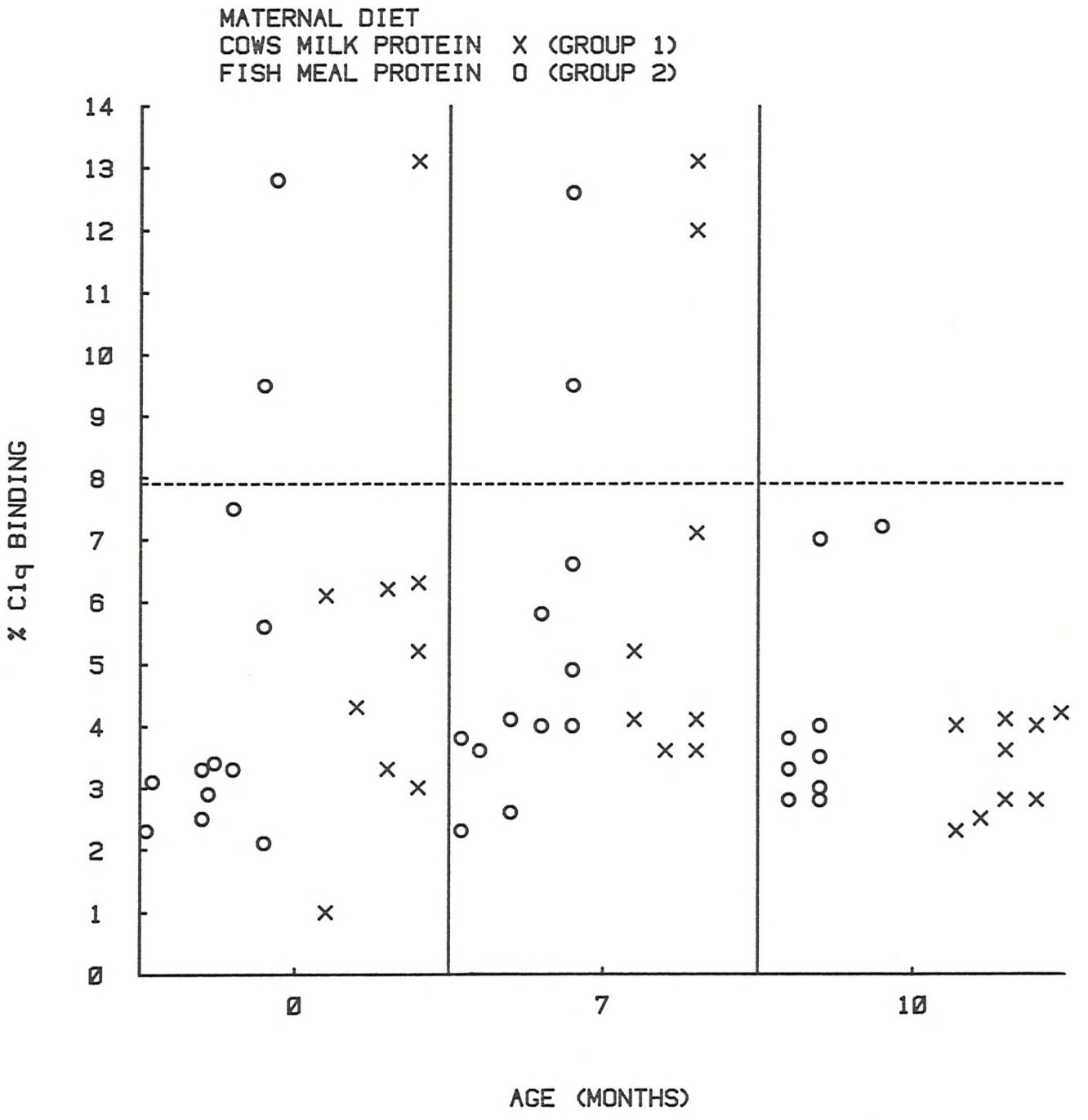


Figure 7.14 INDIVIDUAL SERUM TOTAL IMMUNE COMPLEX RESULTS AT WEANING AND AT 7 AND 10 MONTHS POST-WEANING.

X: GROUP 1. DAMS EXPOSED TO COWS MILK.
REGRESSION COEFFICIENT = -7.65.
O: GROUP 2. DAMS NOT EXPOSED TO COWS MILK.
REGRESSION COEFFICIENT = -5.37.

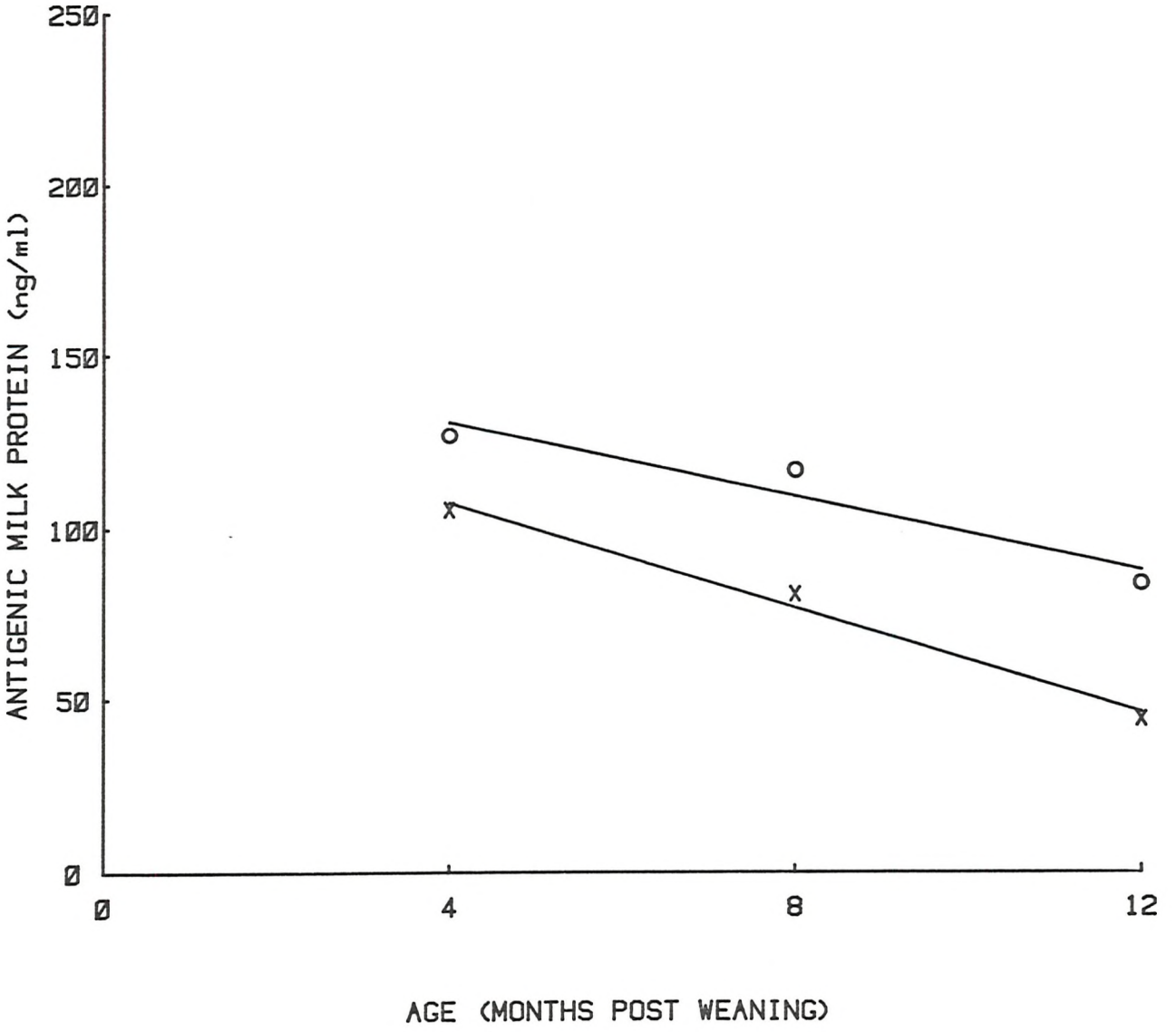


Figure 7.15 THE EFFECT OF MATERNAL EXPOSURE TO COWS MILK PROTEIN ON SERUM CONCENTRATIONS OF ANTIGENIC MILK PROTEIN. (EXCLUDING MEASUREMENTS AT WEANING).

Discussion

The mean concentration of circulating anti cows milk protein lgG in the rabbits of Group 2 (breeding stock had not been exposed to milk) increased during the experimental period, thus confirming that rabbits produce an increase in specific serum antibodies when introduced to a novel dietary protein at weaning. In contrast, systemic tolerance to milk protein was observed in the group of rabbits in which the maternal breeding stock had been exposed to milk protein (Group 1). However, a significant difference between the antibody levels of the two groups was not observed until 3 months after the introduction of the cows milk diet (Table 7.3). This may also explain the lack of a significant difference in serum antibodies in rabbits from dams exposed to milk, compared with those not exposed to milk in Group 1 and Group 2 of Experiment 3b, when introduced to a diet containing 10% DSM. The development of a good humoral response or systemic tolerance to a dietary protein may not only depend on the quantity and source of ingested protein but also on the length of time that the protein is consumed. In this experiment the difference between the systemic immune response of Group 1 and Group 2 increases during the experimental period.

Although the level of specific serum antibodies increased significantly in Group 2 during the experimental period (Fig. 7.9), there was no evidence of abnormal levels of circulating immune complexes (Fig. 7.14) or an increased amount of aortic atheroma compared with Group 1. (Table 7.10). It therefore appears that the magnitude of the immune response to milk protein in Group 2 was insufficient to influence the development of atherosclerosis. The lack of disease in the rabbits of Group 2 differs from the result observed in a similar experiment in which a diet based on soya protein was fed to rabbits. In this experiment the rabbits which were not made tolerant to soya protein developed increased levels of specific serum antibodies and immune complexes and developed more atherosclerosis than a similar group of rabbits made tolerant to the protein *(Gallagher et al., 1983)*. The difference in the final result of the two studies may be because of a less dramatic immune response produced by the ingestion of milk protein compared with soya protein which has been reported in rabbits *(Pathirana et al., 1981)*.

Analysis of the serum for antibodies specific for the individual milk proteins (casein, β lactoglobulin, α lactalbumin and BSA) showed

that Group 1 were tolerant to β lactoglobulin, α lactalbumin and casein. A significant rise in anti BSA lgG was observed in this group at month 8 after the introduction of the cows milk diet (Fig. 7.13). Group 2 developed significant levels of specific serum antibodies against each of the milk proteins by 4 months after the introduction of the milk protein diet (Fig. 7.10, 7.11, 7.12 and 7.13). However, a continued increase in specific serum lgG antibodies was only observed against casein (Fig. 7.10). It is difficult to make any comparison between the magnitude of the immune response produced by the individual milk proteins as the results are expressed as percentages of different reference samples. However, the systemic immune response does not appear to be proportional to the quantity of specific protein ingested. The approximate quantity of specific milk proteins ingested by each rabbit daily is given in Table 7.13.

Studies in guinea pigs have found that antibodies are mainly produced against β lactoglobulin, following the ingestion of unheated whey *(Heppell et al., 1984)*. The ability of β lactoglobulin to stimulate the production of specific serum antibodies may be due to its resistance to proteolysis *(Bleuminck, 1979)*.

The systemic tolerance to cows milk proteins that was apparent in Group 1 may have been due to the presence of an efficient mucosal immune system. Dietary proteins fed to pregnant rats have been found to reach the circulation of the foetus *(Dahl et al., 1984)*, and food proteins have been detected in maternal milk *(Jacobsson & Lindberg, 1983)*. *Rothberg (1969)* has demonstrated that antigenic stimulation rather than gestational age is important for the development of an immune response in the neonatal infant. Alternatively, maternal slgA specific for cows milk protein may have been acquired from colostrum or milk prior to weaning. Maternal lgA may still be present during the first days after weaning, protecting the gut from antigenic absorbtion.

Cows milk antigen was measured in the serum of both groups. The decline in antigen was similar in both groups during the experimental period (Fig. 7.15). However, at weaning, the concentration of specific antigen was significantly greater in Group 2, compared with Group 1 (Table 7.9). The high level of circulating specific antigen in the serum of Group 2 at weaning is likely to be maternal milk protein as these rabbits have not been exposed to cows milk protein. A similar

TABLE 7.13

Approximate Quantity of Specific Milk Protein Ingested by Each Rabbit (Grams/day)

Specific Milk Protein	Grams Consumed per Day
Casein	39.9
β Lactoglobulin	1.27
α Lactalbumin	3.59
Bovine Serum Albumin	0.348

finding has been observed in an unpublished study *(Harris & Gibney, unpublished communication)*. Experiment 4b indicates that the antigen detected at weaning is not the milk protein casein. There was no increase in specific antigen after the introduction of the cows milk diet, but a steady decline in specific antigen concentration was observed. The decline in specific antigen in Group 2 coincides with the development of a systemic humoral response and possibly with an effective mucosal immune system.

CHAPTER 8

FINAL DISCUSSION

Final Discussion

The results presented in this thesis illustrate that the systemic immune response produced by the ingestion of a dietary protein depends on a number of factors.

The first experiment (Chapter 3) shows that the immune response produced by the more mature rabbit is more dramatic than that produced by the weanling, following the ingestion of a novel dietary protein. This suggests that absorbtion of macromolecular protein across the gastrointestinal barrier in sufficient quantities to stimulate serum specific antibodies is probably not simply a post weaning phenomenon. Systemic tolerance to the protein was observed in the offspring when the dams were exposed to the protein during pregnancy and lactation. This form of tolerance was more readily produced when the dietary protein was introduced to the older offspring, compared with the weanling. Tolerance to dietary antigen in the weanling animal was found to depend on the quantity of protein in the diet and the duration of exposure to that protein (Chapter 7).

The importance of dose and source of a particular protein on its ability to stimulate the production of specific serum antibodies was confirmed in experiment 4. Both these factors will influence the amount of macromolecular protein that reaches the circulation and therefore the number of antigenic determinants present. Stimulation of a systemic immune response will not only depend on the quantity of protein reaching the circulation in a macromolecular form, but also on the binding dynamics of accessible antigenic determinants. *Aas (1984)* found that only a few amino acid side chains were necessary for producing the potency of the major allergen in cod fish (allergen M), but the distance between the amino acid side chains was critical and could be maintained by other amino acids which act only as inert frame-works or spacers.

Although the quantity of protein which reaches the circulation in an antigenic form will depend on the amount of protein ingested (experiment 4a) and the physical properties of the protein (experiment 4b) such as resistance to proteolytic enzymes, it will also depend on the mucosal immune response to that protein.

The systemic tolerance produced to a dietary protein in the offspring from dams which had been exposed to the protein during pregnancy and

lactation may be due to the presence of maternal antibodies. The rabbit receives maternal antibodies in the form of IgG in utero which has been found to suppress the immune response in the newborn *(Leiper & Solomon, 1974)*. The suckling animal will also receive maternal secretory IgG via the colostrum and milk. Maternal antibodies may have been involved in the production of hyporesponsiveness in the offspring, but the systemic tolerance was observed in the offspring which were not introduced to the protein until 12 weeks post-weaning, suggesting other mechanisms are involved. The tolerance may have been acquired from the passage of small quantities of antigen from the mother either in utero or via the colostrum and milk. Experiments in mice suggest that absorbtion of antigen via the colostrum and milk results in tolerance *(Auerbach & Clark, 1975; Halsey & Benjamin, 1976)*. A recent study indicates that exposure to an antigen either in utero (via transplacental passage) or postnatally (via the colostrum) generated the production of primary effector suppressor T cells and the induction of memory in the suppressor T cell population *(Fazekas de St. Groth et al., 1984)*. The presence of memory suppressor T cells may explain the specific systemic tolerance observed when the protein was not introduced to the rabbits until 6 or 12 weeks post weaning (Chapter 3). Thus, the final response to an immunogen challenge may depend on the number of effector T suppressor cells and the timing of their appearance in relation to T helper and B cells. The rapid generation of sufficient numbers of suppressor T cells may be necessary to ensure that most B cells and possibly T helper cells are prevented from becoming activated and this may result in tolerance to a particular antigen. *Richman et al. (1981)* have reported the appearance of antigen specific IgA helper T cells in the Peyers Patch after the oral administration of ovalbumin together with IgG specific suppressor T cells. The presence of suppressor T cells for IgG and helper T cells for IgA may influence the specific immune response produced later in life. Local immunity in the form of secretory IgA may reduce the absorbtion of antigen *(Swarbrick et al., 1979)*, and immune complexes containing IgA have been suggested to produce systemic hyporesponsiveness, possibly by paralysing B cells *(Andre et al., 1975)*.

A small increase in serum specific antibodies was observed to milk protein in the offspring of dams exposed to milk during the first few weeks after weaning (Fig. 7.2). This may have been due to immaturity

of the mucosal immune system.

An efficient mucosal immune system possibly prevented the absorbtion of measurable quantities of antigenic protein following acute ingestion of cows milk or goats milk in the majority of adult volunteers in experiment 6. The absence of serum specific antigen on Clq binding immune complexes by most of the individuals after consuming goats milk and the presence of serum antibodies directed against goats milk protein in all the subjects suggests that goats milk protein may not have been treated as a novel protein but was recognized by antibodies directed against cows milk protein.

The majority of adults have only low levels of circulating antibodies to common food proteins. This tolerance may have been acquired in utero or during the neonatal period. Small quantities of cows milk protein and other proteins have been shown to cross the maternofoetal barriers in the rat *(Dahl et al., 1984)* and dietary proteins have also been detected in human breast milk by *Kilshaw & Cant (1984)*. They suggest that selective transport of protein across the mammary epethelium may occur, possibly complexed with dimeric lgA. These trace quantities of dietary protein in breast milk may be of immunological significance in the newborn.

The systemic tolerance observed in the rabbit produced by maternal exposure to the specific dietary protein was not observed in the mouse. The presence of circulating maternal antibodies may have masked any unresponsiveness in the offspring (experiment 5a and 5b). However, tolerance was not apparent at an age when the maternal antibodies would have declined (experiment 5c).

Although tolerance has been produced in mice by maternal immunization *(Auerbach & Clark, 1975; Halsey & Benjamin, 1976)*, other studies have confirmed that ingestion of a soluble protein by neonatal rabbits tends to produce tolerance *(Rieger et al., 1981)*, whereas ingestion of a soluble protein by neonatal mice does not *(Strobel et al., 1984)*. This difference may be due to the quantity of protein that the neonate is exposed to. The mouse may be exposed to greater quantities of antigenic protein compared with the rabbit. The rabbit receives passive immunity in utero, whereas the mouse absorbs maternal lgG until 17-19 days post partum when gut closure occurs. Thus the intestine of the mouse during the first few weeks after birth is probably more permeable than the

intestine of the neonatal rabbit. If the quantity of antigenic protein that the neonate is exposed to is important in determining whether tolerance or sensitization to a particular protein occurs, it may explain the observation that food allergic diseases are more common in bottle rather than breast fed infants.

Although the quantity and source of antigen, the age of the animal and the route of antigen exposure and the presence of maternal antibodies may all influence the immune response produced by the specific antigen. It also appears to be an individual response resulting in large individual variations. *Stokes et al. (1983)* have shown that genetic differences exist in the immune response to ingested antigens and they suggest that orally induced tolerance and immune exclusion are inherited independently.

The difference in the individual response may contribute to the different vulnerability to food allergy in unphysiological circumstances such as infant feeding. However, in order to understand the mechanisms involved in adverse reactions to food proteins which are mediated by the immune system, it is necessary to understand the normal response to ingested dietary protein. It appears that the normal healthy adult has an efficient gastrointestinal barrier, preventing excessive absorbtion of antigenic material resulting in systemic tolerance to common dietary proteins. This tolerance may be influenced by the maternal diet as in the rabbit and by infant feeding practices. Further research into the importance of these factors in man may help to establish the mechanism of tolerance observed in the majority of the population and therefore provide an understanding of diseases mediated by immune mechanisms.

APPENDIX I

TABLE 3.9 (i)

Individual Antimilk Protein Antibodies in the Serum of New Zealand White Rabbits from Dams not Exposed to Milk Protein

A diet containing 10% DSM was introduced at weaning (Group 1), 3 weeks post weaning (Group 2) and 12 weeks post weaning (Group 3). The serum was diluted 1:200 with PBST and the results are expressed as the optical density reading at 405 nm.

Individual Rabbit	Time (weeks after introduction of DSM)		
Group 1	0	3	6
1a	0.187	0.984	0.846
1b	0.10	-	0.551
1c	0.24	1.053	1.135
1d	0.09	0.265	0.980
1e	0.10	0.43	0.682
1f	0.214	0.94	1.00
Group 2			
2a	0.536	1.10	1.81
2b	0.413	1.434	2.066
2c	0.256	0.514	0.716
2d	0.424	0.505	0.732
2e	0.342	1.34	1.099
2f	0.258	0.654	0.868
Group 3			
3a	0.258	0.908	1.428
3b	0.332	1.05	0.905
3c	0.380	0.786	1.471
3d	0.206	0.680	1.476
3e	0.235	0.848	1.322

APPENDIX I

TABLE 3.9 (ii)

Individual Antimilk Protein Antibodies in the Serum of New Zealand White Rabbits from Dams Exposed to Milk Protein

A diet containing 10% DSM was introduced at weaning (Group 1), 3 weeks post weaning (Group 2) and 12 weeks post weaning (Group 3). The serum was diluted 1:200 with PBST and the results are expressed as the optical density reading at 405 nm.

Individual Rabbit	Time (weeks after introduction of DSM)		
	0	3	6
Group 1			
1a	0.201	0.370	0.479
1b	0.132	1.66	1.75
1c	0.328	0.922	0.913
1d	0.287	1.436	1.26
1e	0.506	0.88	1.00
1f	0.282	0.979	1.063
Group 2			
2a	0.48	1.27	1.431
2b	0.88	1.824	1.48
2c	0.458	0.963	0.358
2d	0.433	1.00	1.36
2e	-	0.589	0.488
2f	0.250	0.578	0.342
Group 3			
3a	0.44	0.665	0.855
3b	0.262	0.27	0.625
3c	0.263	0.362	1.082
3d	0.457	0.70	0.989
3e	0.40	0.347	0.699
3f	0.27	0.88	1.075

APPENDIX II

Buffered Solutions used in Assay Methods

1. Bicarbonate - Carbonate Buffer pH 9.6 (ELISA coating buffer)

To 250 ml of 50 mM sodium bicarbonate add enough 50 mM sodium carbonate until pH = 9.6. Add 0.02% sodium azide.
Lasts 7 days at $4^{o}C$.

2. Phosphate - Buffered Saline pH 7.3

To 1 litre of distilled water add:

- 8.0g sodium chloride
- 0.2g potassium chloride
- 1.15g disodium hydrogen phosphate (anhydrous)
- 0.2g potassium dihydrogen phosphate

(Check pH).

3. Phosphate-citrate Solution (Stop solution in ELISA)

To 88.2g tri-sodium citrate add 850 mls distilled water, pH with phosphoric acid to pH 2.85.
Finally make up to 1 litre with distilled water.

4. Borate Buffer

Add to 1 litre of distilled water:
6.18g Boric acid (0.1M)
9.54g Disodium tetraborate (0.026M)
4.38g Sodium chloride (0.075M)

5. Veronal Buffered Saline pH 7.6

Stock Solutions:

a) 85g sodium chloride with 3.75g sodium diethyl barbituric acid made up to 1.4 litres in doublt distilled water.
b) 5.75g diethyl barbituric acid dissolved in 500 ml hot double distilled water.
c) 20.3g magnesium chloride in 50 ml double distilled water added to 30 ml of 1M calcium chloride. Make up to 100 ml in double distilled water.

1) Solution (a) and (b) mixed and cooled to room temperature.

2) 5 ml of solution (c) are added.

3) Final volume adjusted to 2 litres with double distilled water and stored at $4^{o}C$.
This concentration should be diluted x 5 before use.

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