UNIVERSITY OF SOUTHAMPTON

THE INTERACTION OF VITAMIN A DEFICIENCY AND ROTAVIRUS INFECTION

A thesis submitted for the degree of
Doctor of Philosophy

by

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To my parents

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ABSTRACT

FACULTY OF MEDICINE

DEPARTMENT OF NUTRITION

Doctor of philosophy

THE INTERACTION OF VITAMIN A DEFICIENCY AND ROTAVIRUS INFECTION

By- Faruk Ahmed

We have established an experimental model of vitamin A deficiency in the mouse. Animals fed an appropriate diet showed greatly reduced circulating vitamin A levels and depleted liver vitamin A stores after 12 weeks. Rotavirus infection produced marked histopathological changes in the gut of deficient mice when compared with normal and pairfed controls. The spleen weight was increased in deficient infected animals which also showed altered rotavirus antibody levels measured by the ELISA technique. Goblet cell number in the villi was reduced in vitamin A deficient animals and this may have contributed to the susceptibility of adult deficient mice to rotavirus infection. Cell mediated immunity, measured as the delayed type hypersensitivity response to Picryl chloride was reduced in animals with vitamin A deficiency.

In parallel studies the effect of a reduction in total food intake was measured in mice receiving up to 50% less diet for 7 or 12 weeks. Although some histological abnormalities were detected in the gut of the animals grossly deficient in the diet, rotavirus infection did not produce intestinal damage beyond this. Delayed hypersensitivity and anti rotavirus antibody levels following oral challenge were normal in animals on a reduced intake.

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µg	= Microgram.
mg	= Milligram.
g	= Gram.
kg	= Kilogram.
µ	= Micron.
cm	= Centimetre.
mm	= Millimetre.
nm	= Nanometre.
ml	= Millilitre.
dl	= Decilitre.
µl	= Microlitre.
N	= Normal.
M	= Molar.
mM	= Millimolar.
MW	= Molecular weight.
O.D.	= Optical density.
I.U.	= International units.
ppm	= Parts per million.
C	= Centigrade.
%	= Percent.
SEM	= Standard error of the mean.
Ig	= Immunoglobulin.
HCl	= Hydrochloric acid.
SRBC	= Sheep red blood cell.

CHAPTER ONE

INTRODUCTION

CHAPTER ONE

General introduction

Malnutrition is one of the most common problems in developing countries. Malnutrition and infection are closely related. Malnourished individuals are more susceptible to the development of infectious diseases and these diseases tend to be more severe when they occur in the malnourished. It is difficult to predict the consequence of infectious diseases and malnutrition in the same subject from a knowledge of either alone.

The interaction of malnutrition and infection results in a marked increases in mortality and morbidity among infants and young children in developing countries (Scrimshaw et al.,1968). Malnutrition, whether due to deprivation of a single nutrient or to more generalized protein-energy malnutrition, has frequently been associated with diminished immunocompetence and lowered resistance to infection (Gross and Newberne,1980; Beisal et al.,1981). In recent years, the awareness and growing concern of the relation between nutrition and immunity has been increased.

Vitamin A, in addition to its general function in the maintenance of growth and development, is an essential nutrient for the anatomical and functional integrity of the epithelium and the mucosa (Olson,1972). In tracheal tissues, for instance, there is a tendency to keratinization, whereas in the gut there is a reduction in the number of mucus-secreting cells in vitamin A deficiency. At a functional level, Vitamin A deficiency is associated with increased susceptibility to infection, especially those infections involving mucosal surfaces (Krishnan et al.,1976; Scrimshaw et al.,1968; Bang and Bang,1969). Vitamin A deficiency also results in an increased prevalence and severity of infections beyond the mucosal surface. It has been reported that vitamin A deficiency is associated with the

atrophy of lymphoid organs, impaired cell mediated and humoral immune response, and decreased lymphoid cell infiltration into submucosal areas such as the gastrointestinal and respiratory tract (Scrimshaw et al.,1968; Krishnan et al.,1976; Krishnan et al.,1974; Bang et al.,1972). Conversely, high doses of vitamin A are known to have adjuvant effects (Jurin and Tannock,1972). Others have claimed no significant changes in cell mediated and humoral immune responses, either due to vitamin A deficiency or by using excess vitamin A in the diet (Kutty et al.,1981; Ways,et al.,1980; Brown et al.,1980). In rationalizing these conflicting results, it should be kept in mind that the vitamin A deficient animals or patients studied by most investigators are likely to have superimposed secondary inanition, an unavoidable complication. Secondary nutrient deficiencies can in themselves lead to changes in immune response unrelated to vitamin A deficiency. Therefore the exact role of vitamin A deficiency on the immune response remains to be elucidated.

The following sections are concerned with vitamin A deficiency and the immune system, and cover the biochemistry of vitamin A, the immune system in general and the background literature of vitamin A deficiency or excess in relation to immune function. The objective of the present series of studies is outlined.

1.1. Sources of vitamin A :

Vitamin A is one of the fat soluble vitamins and is a unique vitamin in that it exists in the plant world only in the form of precursor compounds, carotenoids, (provitamins). The number of known carotenoids is more than 500, but the vitamin A active compounds are only a fraction of the total. There are only 50 compounds in the carotenoid family with vitamin A activity (Olson,1984). Carotenes are found in abundance in carrots and green leafy vegetables. The best sources of preformed vitamin A are liver, milk and whole eggs. Table: 1.1 shows the vitamin A and β-carotene content of some foods (Bondi and Sklan, 1984).

Table: 1.1

Vitamin A and β-carotene contents of some foods (µg/g) (Bondi and Sklan, 1984).

Food	Vitamin A	β-Carotene
Milk (cow,summer)	0.03-0.05	0.02-0.04
Milk (cow,winter)	0.02-0.04	0.005-0.02
Milk (human)	0.05	0.027
Eggs (without shells)	0.2-1.5	2.3
Liver (bovine)	1.5-9.0	0.5-2.0
Liver (chicken)	2.0-20.0	5.0
Cod liver oil	130.0	
Whale liver oil	1300.0	
Apricot		13.0-21.0
Avocado		0.4-0.7
Carrots		12.0-72.0
Lettuce		2.0-3.3
Oranges		1.4-2.7
Papaya		1.2-5.0
Pepper,red		11.0-33.0
Pepper,green		1.2-1.5
Tomatoes		5.0-9.0

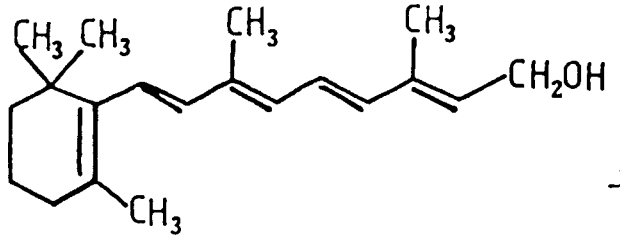
1.2. Chemical structure of vitamin A and its activity :

Figure:1.1 shows the structural formula of vitamin A and its related compounds. The all-trans retinol molecule shows the highest vitamin A activity. Some changes at the end of the retinol chain, for example retinoic acid, have growth promoting activity but do not maintain visual pigment (Dowling and Wald,1960). Thompson et al (1964) showed that retinoic acid could not satisfy the requirement for vitamin A for reproduction in rats. Modifications elsewhere other than at the end of the retinol chain usually abolished activity or reduced it to a negligible amount (Ames 1965; Pitt,1965).

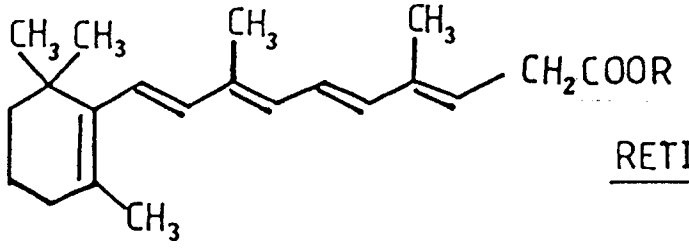
1.3. Absorption and metabolism of vitamin A :

Vitamin A enters the animal and human organism through the diet in the form of provitamin A from plants or as preformed retinol or as retinol derivatives from animal tissues. In the stomach, retinyl esters and various carotenoids are released from food by proteolytic activity. During intestinal absorption, carotenoids are largely converted into vitamin A and the conversion mainly occurs in the proximal portion of the rat small intestine (Huang and Goodman,1965). Huang and Goodman (1965) also confirmed that the rat intestine absorbs very little intact carotene. However, Goodman et al (1966) have demonstrated that humans can absorb a significant amount of intact β-carotene. The conversion of β-carotene into vitamin A involves, first, the central cleavage of β-carotene into two molecules of retinal (Goodman and Huang,1965). The newly formed retinal is then reduced to retinol which in turn is esterified with fatty acids and transported via the intestinal lymphatics (lymph), largely in association with lymph chylomicrons (Huang and Goodman,1965). The intestine is the major site of conversion of carotenoids to vitamin A but cleavage can also occur in the liver (Olson and Hayaishi,1965).

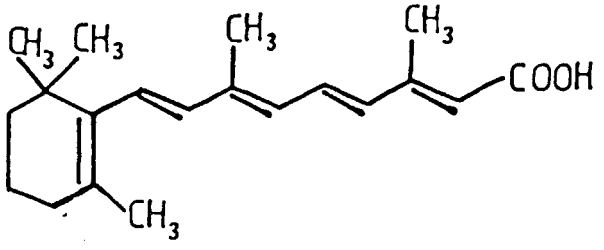
Free vitamin A alcohol either from dietary sources or resulting from hydrolysis of dietary vitamin A ester passes the



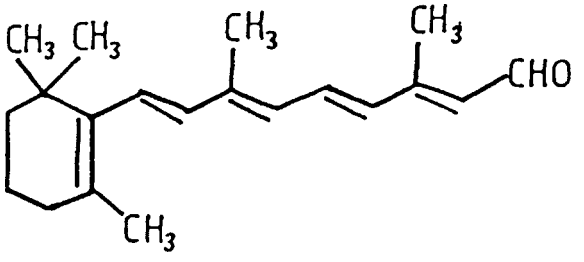
RETINOL



RETINYL ESTER



RETINOIC ACID



RETINALDEHYDE

Figure: 1.1 The structural formula of vitamin A and its related compounds. R= fatty acyl group.

mucosal cell membrane and is absorbed by the intestinal cells, in which it is reesterified to fatty acid and than transferred to the lymphatic circulation (David and Ganguly,1967; Huang and Goodman,1965). Absorption of carotene and retinyl esters is dependent on intestinal bile salts, dietary proteins, fats and vitamin E (Moore,1957).

The transport of vitamin A following its absorption from the intestine (to the liver) occurs mainly as retinyl esters within the lipid phase of low density lipoproteins (Ganguly,1960), but the retinol is transported in plasma from the liver in 1:1 M combination with retinol binding protein (RBP). Holo RBP (complex of retinol and RBP), is also combined with thyroxine binding prealbumin (TBPA) in a 1:1 M ratio (Reviewed by Wolf,1984). The RBP-retinol complex protects and stabilizes the vitamin, makes it soluble and transportable in plasma. The combination of TBPA with holo-RBP increases the stability of the retinol bound to RBP (Raz et al.,1970). A third binding site of holo-RBP enables it to bind to the membrane receptors of target cells so that the retinol can be released to cross the membrane. For intracellular transport of retinol, it must bind to cytosolic retinol binding protein, CRBP (Bashor et al .,1973; Chytil and Ong,1978), for delivery to its site of action within the cell.

Between 70 to 90% of the body's vitamin A is stored in the liver. Approximately 80% of the total liver vitamin A is stored in the stellate cell or fat storing cells and 20% is present in parenchymal cells (Hendriks et al.,1985; Blaner et al.,1985; Batres and Olson,1987). Little change is observed in the pattern of distribution over a wide range of liver vitamin A stores. However as vitamin A reserves fall to a low level (1.2 µg/g) 83.5% is present in the parenchymal cells and 16.5% in stellate cells (Batres and Olson,1987).

The second most important organ for vitamin A storage is often said to be the kidney. However, this is probably not true storage, it is known that a portion of holo-RBP filters through the glomeruli and is reabsorbed by the tubules, where retinol is liberated while RBP is degraded (Vahlquist,1972). Pigment epithelium of retina

is another storage site for retinol. Here the retinol arrives from the circulation and is esterified and stored in lipid droplets (Reviewed by Wolf,1984).

Figure: 1.2 shows a schematic summation of the vitamin A metabolism presented by Underwood et al (1979).

1.4. Catabolism :

Vitamin A, in the liver or in the target tissue undergoes metabolism for elimination and for functional activation. The first metabolic product of retinol, for both the activation and elimination pathways, is retinoic acid (Wolf,1984). Retinoic acid is capable of maintaining normal growth and a healthy epithelium but is inactive for normal vision. 13-cis retinoic acid is the next metabolic step which finally conjugates with glucuronic acid to form 13-cis retinoic acid glucuronide (Zile et al.,1982). Glucuronides are the normal elimination product. Some of the retinoic acid is converted to the glucuronide by another pathway. Both trans-retinoic acid and 13-cis retinoic acid are converted to 13-cis-4-oxoretinoic acid through 13-cis-4-hydroxy retinoic acid (Frolik et al.,1980) which has no biological activity. The 4-oxo derivative can then be glucuronidated to form 13-cis-4-oxoretinoyl glucuronide and 13-cis-retinoyl glucuronide (Frolik et al., 1981).

5,6-epoxy retinoic acid is a metabolite of retinoic acid of a second oxidation pathway (Napoli et al.,1978) and 5,6 epoxy retinoic acid is further metabolized to 5,6-epoxy retinoyl glucuronide for the elimination pathway (Napoli et al.,1982). A summary of the known metabolic reactions of retinol which lead to the excretion of vitamin A including the active forms of all trans and 13-cis retinoic acid are shown in figure: 1.3.

1.5. Vitamin A homeostasis :

The liver is the organ where vitamin A is stored. The release

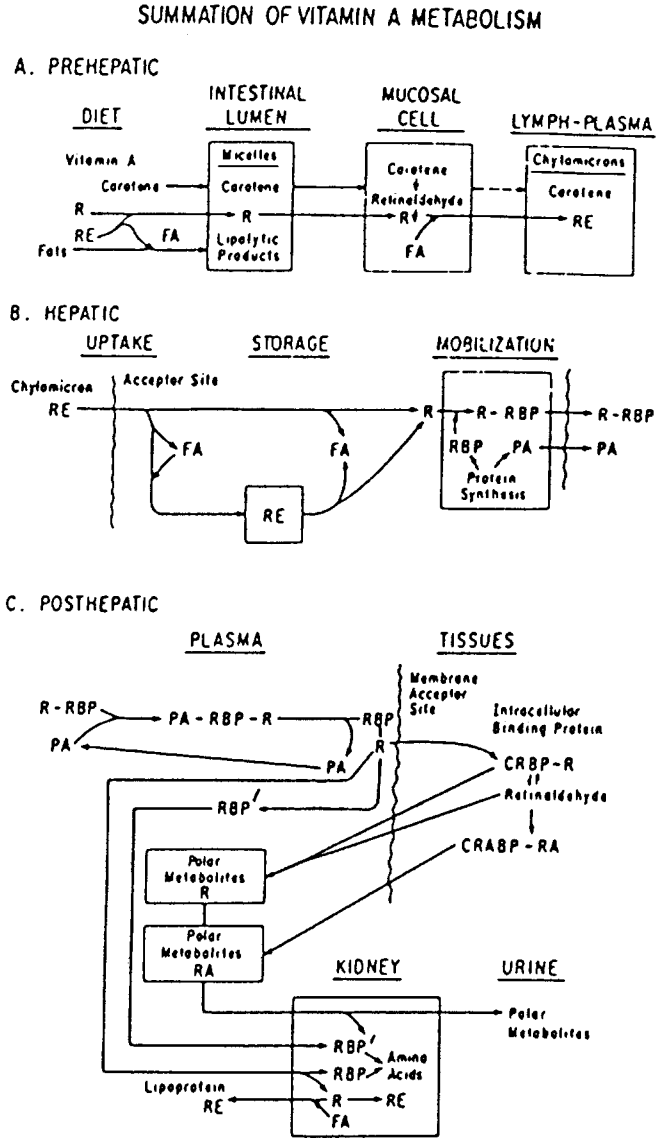


Figure: 1.2 A schematic summation of the metabolism of vitamin A; prehepatic, hepatic and posthepatic. (Underwood et al., 1979). R=retinol, RE=retinyl ester, FA=fatty acid, PA=prealbumin, RBP=retinol binding protein. CRBP=cellular retinol binding protein. CRABP=cellular retinoic acid binding protein. RBP'=partially denatured retinol binding protein.

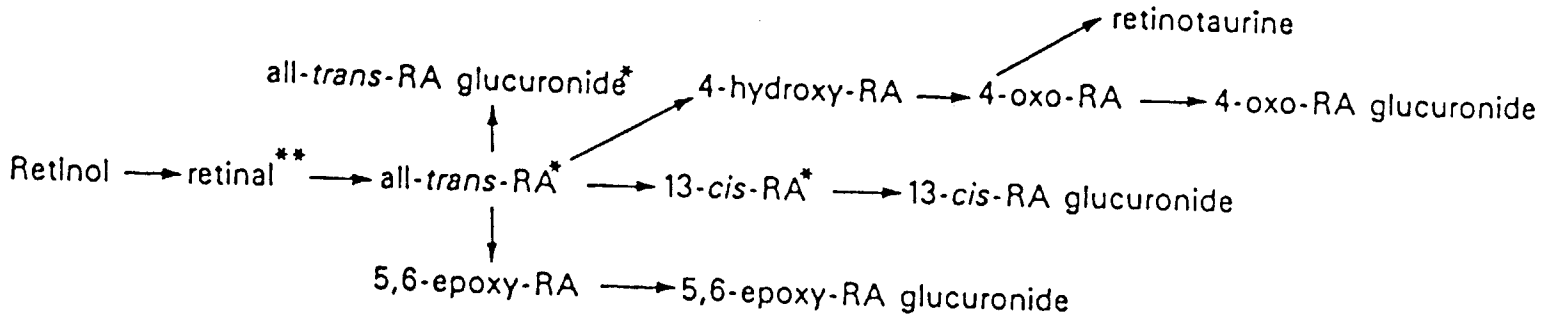


Figure: 1.3 Metabolic pathways of retinol leading to elimination. RA=retinoic acid. *Suggested as possible active metabolites of vitamin A. **Possible intermediate; not isolated except in the eye. (Wolf, 1984).

of vitamin A from the liver is controlled, and optimum circulating levels for the tissues needs are maintained. Thus the level never becomes excessive in the tissues which may lead to toxic effects. Plasma retinol levels are constant despite great variation in dietary supply and in liver reserve stores (Wolf,1984). There is a homeostatic mechanism that maintains plasma retinol within a range that is normal for a given species, individual and circumstances.

Underwood et al (1979) have provided evidence that the level of plasma retinol and the rate of its release from liver, is determined by the vitamin A needs of extrahepatic tissues. They suggested that the lack of dietary supply of the vitamin A is compensated by a regulating mechanism that releases enough of the vitamin A from the liver reserve to maintain plasma levels at the normal homeostatic set point. Provided that the liver reserve is above 5 µg/g wet weight, the reserve can supply the tissue needs adequately. They also suggested that following supplementation with retinoic acid, the plasma set point for retinol shifts downwards and lowers mobilization from the liver. As retinoic acid takes over some of the functions of retinol, tissue utilization and metabolism of retinol is decreased and thus less retinol is mobilized.

There are a number of regulatory factors that control the homeostatic set point for vitamin A. The most important is the availability of retinol from endogenous and exogenous sources. Serum vitamin A deficiency results in a decline in the level of plasma holo-RBP (complex of retinol-RBP, Muto et al.,1972). The amount of retinol binding protein is another factor which also regulates plasma levels of retinol (Goodman,1974). An adequate amount of dietary protein is known to be the important factor, because amino acids are involved in RBP synthesis (Smith et al.,1973; Muhilal and Glover,1974). Zinc deficiency is also known to be accompanied by a low level of plasma retinol. Zinc has been suggested as another factor which may regulate vitamin A homeostasis (Apgar,1977; Smith et al.,1973a).

1.6. Functions of vitamin A :

1.6.1. Physiological functions :

Vitamin A is involved in a number of physiological functions in animals and humans. It is essential for the stimulation of growth, and proper development of skeletal tissues, for vision and for normal reproduction (Weber,1983). One of the earliest and most important observations concerning vitamin A function is that it controls epithelial differentiation. Wolbach (1954) has suggested that vitamin A deficiency can change the epithelial structure of salivary glands, respiratory tract, genito urinary tract and the conjunctiva and cornea of the eye. He also suggested that the changes involved atrophy, reparative proliferation of basal cells and differentiation of the new cells into a stratified keratinizing epithelium. But there is an exception, namely, the intestinal mucosa, where the epithelium does not keratinize but the number of goblet cells of this tissue decreases considerably (Deluca,et al.,1969). Severe deformations also occur in cartilage and bone during vitamin A deficiency (Mellanby,1938). The liver itself does not undergo any morphological changes (Wolbach,1954a).

1.6.2. Biochemical functions:

Vitamin A is thought to be involved in the biosynthesis of mucopolysaccharides by the mucous epithelium (Wolf and Johnson,1960). The possible mechanism of vitamin A involvement is a coenzymic function. Vitamin A is also involved in the synthesis of glycoprotein in the tracheal respiratory epithelium (Bonanni,et al.,1973), intestinal mucosa (DeLuca et al., 1970), liver (DeLuca et al.,1975) and corneal epithelium (Kim and Wolf,1974). The molecular mode of vitamin A action in glycoprotein synthesis is reviewed well by DeLuca (1977). He suggests that the retinyl phosphate acts as a direct donor of mannose to form mannosyl glycoprotein. Kiorpes et al (1981) have

also shown that the glycosylation of serum α-macroglobulin, a rat serum glycoprotein, was decreased by vitamin A deficiency. But certainly vitamin A deficiency does not affect the synthesis of all glycoproteins. Ovalbumin, a high mannose glycoprotein of chick oviduct, responds to estrogen stimulation, equally in vitamin A deficient and in normal control chicks (Sneider and Wolf,1976). There is an increased level of serum fibronectin in vitamin A deficient rats (Zerlauth et al.,1984) and also increased rates of fibronectin production were observed in liver and hepatocytes prepared from vitamin A deficient rats as compared to pairfed controls (Kim and Wolf,1987).

Pronounced effects of vitamin A deficiency on RNA synthesis in tissues, such as intestinal mucosa, tracheal epithelium and testis were described by Ganguly et al (1980). Tsai and Chytil (1978) have shown that the RNA synthesis in isolated nuclei from liver of vitamin A deficient rats was about 50% lower than the pairfed control. DNA synthesis of epithelial tissues was also found to be decreased in vitamin A deficient rats (Zile et al.,1981). Estrogen primed oviducts of vitamin A deficient immature chicks showed marked inhibition of total DNA, RNA and protein synthesis which was reversed by retinyl acetate administration (Ganguly et al.,1978). In cell nuclei there is a specific retinol binding site and the hepatic nuclei of vitamin A deficient animals have increased specific binding of retinol compared to controls (Chytil and Ong,1979). Omori and Chytil (1982) have shown the relationship between retinol and gene expression with respect to m-RNA and protein synthesis. In retinol deficient rats, the level of poly-adenosine-RNA per DNA (m-RNA) declines. These authors have also shown that the decreased level of poly(A)-RNA per DNA is not due to altered cell populations but is an effect on actual expression of the genome. The most recent work of Kim and Wolf (1987) has shown that vitamin A was involved in the synthesis of a specific protein in the liver at the genomic level. They have also shown that the vitamin A deficient liver cells have 2-4 fold higher level of fibronectin m-RNA.

Weber (1983) has described the involvement of vitamin A in

lipid metabolism. Triglyceride levels in serum of vitamin A deficient rats was decreased to about 20% of normal. The triglyceride rich lipoproteins, such as chylomicrons and low density lipoproteins of vitamin A deficient rats were found to be decreased about 10 fold. High density lipoprotein, mainly containing phospholipid and cholesterol, decreased by about 40% as compared to control levels.

Vitamin A is most probably involved in multiple functions at the molecular level and it is obviously very difficult to find a common mechanism which could explain the biochemical action of vitamin A. The molecular action of vitamin A clearly requires further investigation.

1.7. Immune system :

The immune system is extremely complicated and exhibits a variety of roles to maintain homeostasis and health by protecting the body from invading foreign materials or organisms. A normally functioning immune system is an effective defense against foreign agents such as pathogenic microbial organisms. The immune system is divided into two functional divisions, namely the innate immune system and the adaptive immune system (Roitt et al.,1985).

1.7.1. The innate immune system :

The innate immunity acts as a first line of defense against infectious agents, and normally acts non-specifically. The innate immune system involves a variety of biochemical and physical barriers, in which phagocytes and mucins play an important role.

1.7.1.1. Mucin :

Mucins are found in mucus, which is usually defined as the viscous fluid lining of the epithelium of the gastrointestinal, respiratory and genitourinary tract (Cook,1972). Mucins are large

glycoproteins (mol.wt. over $1X10^{6}$) containing from 50 to 80% or more by weight of carbohydrate. Chemically, mucins consist of nitrogen containing monosaccharide such as glucosamine or galactosamine. The mucins can be classified into neutral and acid type. The neutral mucins consist of hexosamine and hexose units and do not have free acidic groups. Acid mucins consist of hexosamine units which may be associated with glucuronic acid or sialic acid.

Mucins are synthesized either by the goblet cells lining the mucous epithelium or by special exocrine gland, such as some of the salivary gland. Goblet cells have been considered important in protection of mucosal infection for a number of years (Ackert et al.,1939; Wells,1963). However, only recently has this cell begun to receive much attention with regard to mucosal resistance. Mucus release from the goblet cells can be stimulated by immune complex (Walker et al.,1977) and by antigen in orally immunized animals (Lake et al.,1979).

1.7.2. The adaptive immune system :

The adaptive immune system is specific and involves two types of immune response, the humoral immune response (antibody formation) and the cell mediated immune response. Both forms of the immune response are carried out by cells with and also without antigen specificity (Golub,1981). The cells with antigen specificity are lymphocytes. The cells without specificity are macrophages and antigen presenting cells.

1.7.2.1. Lymphocytes :

Lymphocytes are derived from hematopoietic stem cells of the bone marrow, which are also capable of producing the myeloid tissue (Ford et al.,1966; Wu et al.,1968). Toward the end of 1960's lymphocytes were shown to fall into two classes (Raff,1970; Mitchell and Miller,1968). A population of cells which have been processed by

the thymus, called T cells, and a population of the cells which have not been processed by the thymus, called B cells (Roitt et al.,1969). The lymphoid cell interaction in the development of the immune response is well established. In humoral responses B cells are recognized as precursors of the cells that produce antibody (Miller and Mitchell,1969; Claman and Chaperon,1969). Thymus derived cells or T lymphocytes, on the other hand, cannot produce antibody (Davies et al.,1967) but play an important role in primary antibody responses by cooperating with B cells (Feldmann,1972). Normally T cells collaborate with B cells for the induction of antibody synthesis in response to antigen (Claman et al.,1966; Mitchell and Miller,1968a).

Cellular immunity is thought to be mediated by T lymphocytes apparently without the help of B lymphocytes. T-cells perform many immunological functions. For example, they generate cytotoxic responses to alloantigens (Golstein et al.,1972) and exert helper activity (Miller and Mitchell,1969). There is increasing evidence to suggest that subsets of T lymphocytes interact among themselves (Cantor and Asofsky,1972; Asofsky et al.,1971). Cantor and Boyse (1975) suggested that the maturation of the subclasses of T cells which perform killer or cytotoxic activity can be amplified by another subclass of T cells similar to those which provide helper activity during antibody responses. There are other subpopulations of T cells which suppress the differentiation of B cells to plasma cells or inhibit antibody formation (Gershon,1974), these are termed T suppressor cells.

1.7.3. The lymphoid system :

The lymphoid system is composed of two functionally separate units, the primary and secondary lymphoid organs (McConnell et al.,1981). In mammals, the primary lymphoid organs consist of bone marrow, the thymus and undefined areas corresponding to the bursa of fabricius in birds. The lymph node, spleen and gut associated lymphoid tissues are the secondary lymphoid organs. The progenitor cells of the

bone marrow migrate to either the thymus or the bursal equivalent via the blood, where they undergo differentiation and become morphologically recognizable lymphocytes but are still predominantly nonfunctional (Golub,1981). Lymphocytes from primary lymphoid organs then enter the secondary lymphoid organs, where they become functional.

1.7.3.1. Thymus :

The thymus is the central or primary lymphoid organ responsible for T cell maturation, producing cells which subsequently occupy the T dependent areas of peripheral or secondary lymphoid tissues and also the recirculating lymphocyte pool (Douglas and Ackerman,1977).

The thymus is bilobed. A lobe is composed of thousands of lobules, each containing cortical and medullary components (Rhodin,1974), an outer dense cortex and an inner paler staining medulla. The cortex contains about 85% of the total lymphocytes. These are densely and uniformly packed. The medulla stains more lightly and is less compact than the cortex. The majority of cortical cells are considered to be functionally immature (Konda et al.,1973; Shortman et al.,1975). In contrast, medullary cells exhibit functional maturity. In the thymus, stem cells for T cells start to proliferate and give rise to the various functional T cell subpopulations, such as helper cells, suppressor cells and cytotoxic cells (Cantor and Weissman,1976).

1.7.3.2. Spleen :

The spleen is a peripheral or secondary lymphoid organ. The spleen consists of two major parts, white pulp, which contains aggregates of lymphoid cells and red pulp, composed primarily of blood contained in broad vascular sinuses (Douglas and Ackerman,1977). The white pulp is responsible for the immune response, and has

identifiable structures surrounding a central arteriole. Around this vessel a reticular superstructure supports a cylinder of lymphocytes, the periarteriolar lymphocyte sheath (PALS), which is a thymus dependent area (Weissman et al.,1974), i.e. contains T cells. In and around the PALS area lie lymphoid follicles with or without germinal centres; these are B cell regions. Germinal centres consist of transforming B cells. There is a loose collection of lymphoid cells which encircle both sheath and follicles, called the marginal zone. Plasma cells are found on the outer edge of the marginal zone and within the red pulp. The lymphocyte numbers in different white pulp compartments of the spleen are influenced by antigenic stimulation (Han et al.,1965).

The red pulp of the spleen contains many scattered B cells and a smaller number of T cells. The red pulp also contains macrophages (Witmer and Steinman,1984). The major function of the red pulp is the removal and breakdown of effete cells, especially erythrocytes from the blood.

1.7.3.3. Lymph node:

The lymph nodes are encapsulated round or kidney-shaped organs composed of lymphoid tissue. They are distributed throughout the body, always along the course of the lymphatic vessels. A lymph node is composed of three zones, the cortex, paracortex and medulla (Roitt et al.,1985). The cortex consist of mainly B cells as the lymphocytes present bear surface immunoglobulin (Gutman and Weissman,1972). In contrast the paracortical zone consists mainly of T cells (Parrott et al.,1966). In the medulla, more mature lymphocytes, macrophages and plasma cells are found.

1.7.4. Lymphocyte traffic :

The lymphon is a collective term for primary and secondary lymphoid organs and their constituent cells. Lymphocyte traffic within

the lymphon occurs in two stages. The first stage involves the migration of lymphocytes to and from the primary lymphoid organs and includes the migration of stem cells from bone marrow to the thymus or bursa (or its equivalent).

The second stage is the mature lymphocyte traffic between the secondary lymphoid organ via blood and lymphatic vessels. Figure: 1.4 shows the major traffic patterns of the lymphocyte (Hobart and McConnell,1975).

1.8. Immunoglobulins :

Immunoglobulins represent a group of structurally related proteins having specific antibody activity. Antibodies are found primarily in the γ-globulin fraction of serum. All immunoglobulin molecules consist of a basic common structure consisting of four polypeptide chains. There are two heavy chains (H) and two light chains (L) which are linked by disulphide bridges (Edelman and Poulik,1961). Each chain is made up of a number of domains of constant dimension(Edelman et al.,1969). In each chain the N-terminal domain has greater variation in amino acid sequence than the others and is called the variable region. The other, less variable domains, are called constant regions (Fleischman, et al.,1963). The light chains of immunoglobulin exist in two antigenically identifiable forms. These are called Kappa(κ) and Lamda(λ). A single immunoglobulin molecule has two of the same variety of light chains, either κ or λ chain (Golub,1981). Five classes of heavy chain have been found in humans μ, γ, α, δ and ε. A typical structure of immunoglobulin G is shown in figure: 1.5.

The human immunoglobulins are classified under a nomenclature agreed by the World Health Organization (1964). The classification is based on the primary structure of their respective heavy chains. There are five classes of immunoglobulins, designated as IgG, IgM, IgA, IgD and IgE. The immunoglobulins of the mouse have also been studied extensively. Only the four classes IgG, IgM, IgA and IgE have been

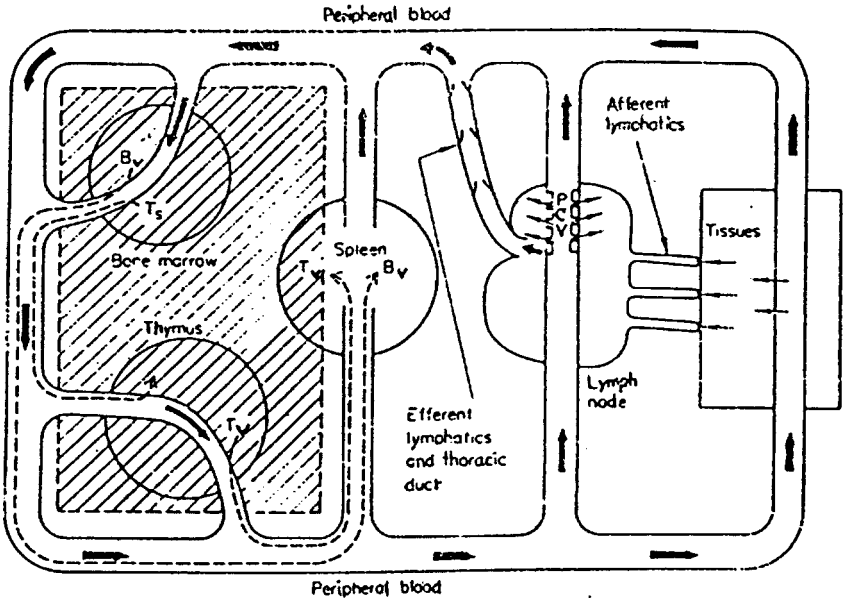


Figure: 1.4 Major lymphocyte traffic patterns within the mature mature lymphon. (Hobart and McConnell, 1975).

Key:

=recurculating lymphocyte pool.

=not part of the recirculating lymphocyre pool.

B_v =virgin B lymphocyte

T_v =virgin T lymphocyte

T_s =lymphoid stem cell

PVC=post capillary vanule.

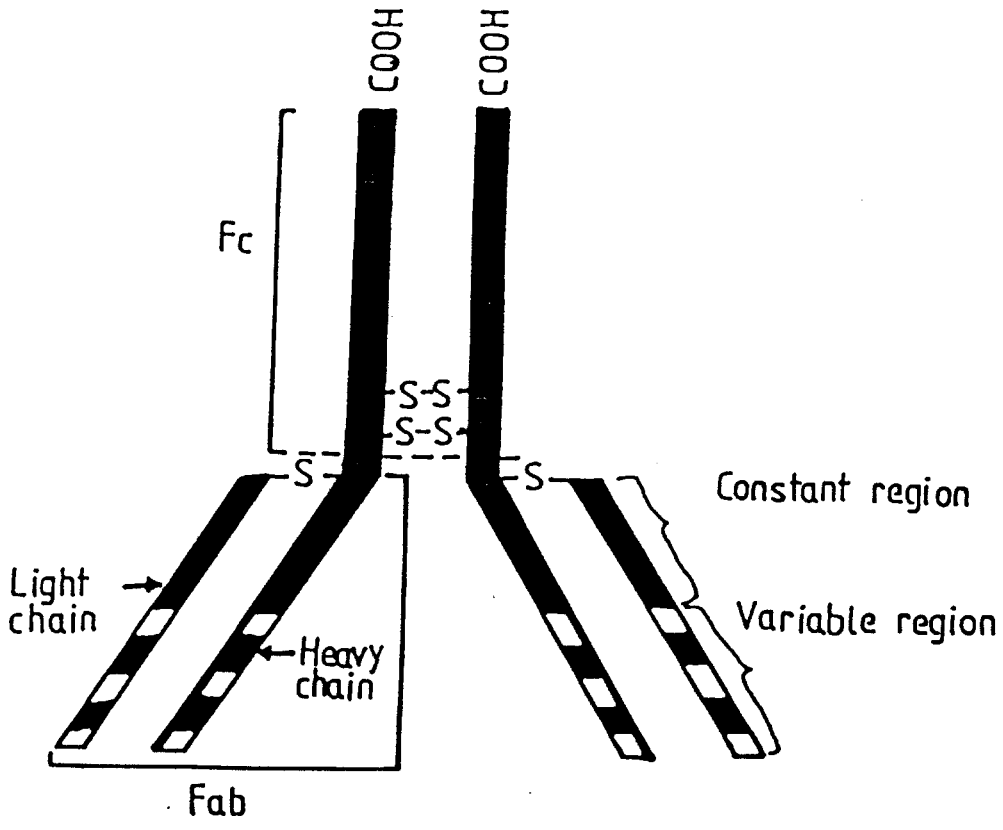


Figure: 1.5 A typical diagrammatic structure of IgG molucule.

Key: Fab= Fragment antigen binding.

Fc = Fragment crystallizable.

documented in mice (Fahey et al.,1964).

1.8.1. IgG:

IgG is the predominent serum immunoglobulin and in normal serum represents approximatly 70-75% of the serum immunoglobulin in man (Roitt et al.,1985). Prolonged antigenic stimulation is required for an IgG response and it is probably the major immunoglobulin to be synthesized during the secondary immune response (Amos,1981). IgG is composed of two heavy chains and two light chains. The heavy chain or γ-chain of the human IgG molecule exists in four different forms, and on this basis four subclasses of IgG, IgG1, IgG2, IgG3 and IgG4 are recognised (Grey and Kunkel,1964).

1.8.2. IgM :

IgM makes between 5 to 10 percent of total serum antibody. IgM antibodies are the first to appear in the circulation following primary antigenic stimulation. Smith and Eitzman (1964) have shown antigen stimulation provokes synthesis of IgM which is then followed by the appearance of IgG antibodies. Each IgM molecule consists of five identical subunits of about 180,000 daltons. Each subunit consisting of two Kappa or Lamda light chains and two μ-heavy chains. The five subunits form a pentamer and are linked by a J chain or joining chain (Amos,1981). In addition to this pentameric form, IgM is also present in serum as a monomer, and B-cell surface IgM is also found in monomeric form. No subclasses of IgM have been isolated.

1.8.3. IgA :

Almost 20 percent of serum immunoglobulin is IgA and mostly in a monomeric form, having a molecular weight of 150,000 daltons. Each molecule consist of two Kappa or two Lamda light chains and two α-heavy chains. The IgA 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 and Grey,1972).

Secretory IgA is a dimer consisting of two monomer subunits (Kagnoff et al.,1973), plus two other non immunoglobulin proteins, one of which is a glycoprotein called secretory component and the other, a polypeptide chain, is termed J chain which holds together the two IgA monomer subunits (Tomasi and Grey,1972).

The antibody response of the intestinal mucosa is mediated by IgA which is produced locally in the lamina propria by plasma cells derived from the B lymphocytes located in the Peyer's patches (Lamm,1976). The role of secretory IgA is to protect the gut from invasion. Secretory IgA has been shown to prevent antigen absorption in the gut (Walker et al.,1974).

1.8.4. IgD :

IgD is the fourth class of immunoglobulin. It has all the common structural features of immunoglobulins. It always exists in a monomeric form. The role of IgD in the immune system is not yet understood. IgD has been demonstrated to be a major class of surface immunoglobulin on human B lymphocytes (Rowe et al.,1973) and on the B lymphocytes of monkey (Martin and Leslie,1979) which has led to the suggestion that it may play an important role in immune recognition and regulation.

1.8.5. IgE :

IgE represents a minor but distinct class of proteins present in the serum of man and higher animals. In man the average concentration is about 0.3 µg/ml (Johansson et al.,1968). IgE is the major class of reaginic antibody (Ishizaka et al.,1966). Tada and Ishizaka (1970) have shown IgE producing plasma cells in the respiratory and gastrointestinal tract which suggests that IgE may be

locally produced.

1.9. Gut immunity :

Most infectious diseases either occur wholly on secretory surfaces or enter the body through a secretory surface. The lymphoid tissue of the gastrointestinal-tract is termed the gut associated lymphoid tissue (GALT). The GALT is comprised of several lymphoid population which include collections of organized lymphoid nodules called Peyer's patches (PP), scattered lymphoid follicles and the lamina propria with lymphocytes and plasma cells. This lymphoid tissue in the intestine is responsible for an immunological response to antigenic challenge (Kagnoff,1987). In general, the Peyer's patches are stimulated by the presence of antigen in the gut and protect the body by secreting immunoglobulins, predominantly IgA (Ganguly and Waldman,1977).

1.9.1. Peyer's patches :

Peyer's patches are the main lymphoid population of the GALT involved in mucosal immunity. They are groups of subepithelial lymphoid follicles located throughout the small intestine. They are separated from the intestinal lumen solely by a thin epithelium which, in contrast to surrounding areas, lacks microvilli (Owen and Jones,1974). Each patch contains three distinct areas, the dome, the follicle and the thymus dependent area (Faulk et al.,1971; Waksman,1973). In the Peyer's patches both T and B cells are present as identified by surface marker characteristics (Levin et al.,1973). B lymphocytes tend to be located in the dome region of the patches where as the T lymphocytes are located in the thymus dependent areas (Waksman,1973) in between the follicles. About eleven to forty percent of the lymphocyte population of the adult mouse Peyer's patch are T lymphocytes and about forty to seventy percent of the population B lymphocytes (McWilliams et al.,1974; Raff et al.,1971).

Both microorganisms and inert particles have been shown to preferentially penetrate into Peyer's patches after oral administration (LaBrec and Formal,1961; Joel et al.,1970). Thus gut antigen would be expected to have ready access to the Peyer's patches. Peyer's patches consist of mature B cells which eventually populate the intestinal lamina propria and other mucosal sites and produce IgA (Craig and Cebra,1971; Cebra et al.,1974).

1.9.2. Mechanisms of gut immunity :

1.9.2.1. Pathways of lymphocyte migration :

When any antigen enters into the gastrointestinal tract, the lymphocytes of the Peyer's patches become stimulated and migrate towards the mesenteric lymph node, then the thoracic duct lymph, and finally to the lamina propria via the circulation (Guy-Grand et al.,1974; Guy-Grand,et al.,1978). Figure: 1.6 showing the lymphocyte migration pathways in the gastrointestinal tract.

1.9.2.2. Antibody response and secretion :

The antibody response in the gastrointestinal tract is performed mainly by IgA that is produced locally by the plasma cells within the intestinal lamina propria (Kagnoff et al.,1973). The transport of this IgA across the intestinal epithelial cell and into the intestinal secretion is shown in figure: 1.7. Secretory component(SC), a glycoprotein, occurs on the basal and lateral plasma membranes of the intestinal surface epithelial cells (Brown et al.,1977) acts as a membrane receptor for IgA and is transported through the intestinal epithelial cells along with IgA. After complexing with dimeric IgA, membrane bound IgA-SC is taken up into the cells and finally transported to the gut lumen. Some dimeric IgA but no SC, can also enter the lymphatics draining the intestine and eventually into the circulation (Vaerman and Heremans,1970). Secretory

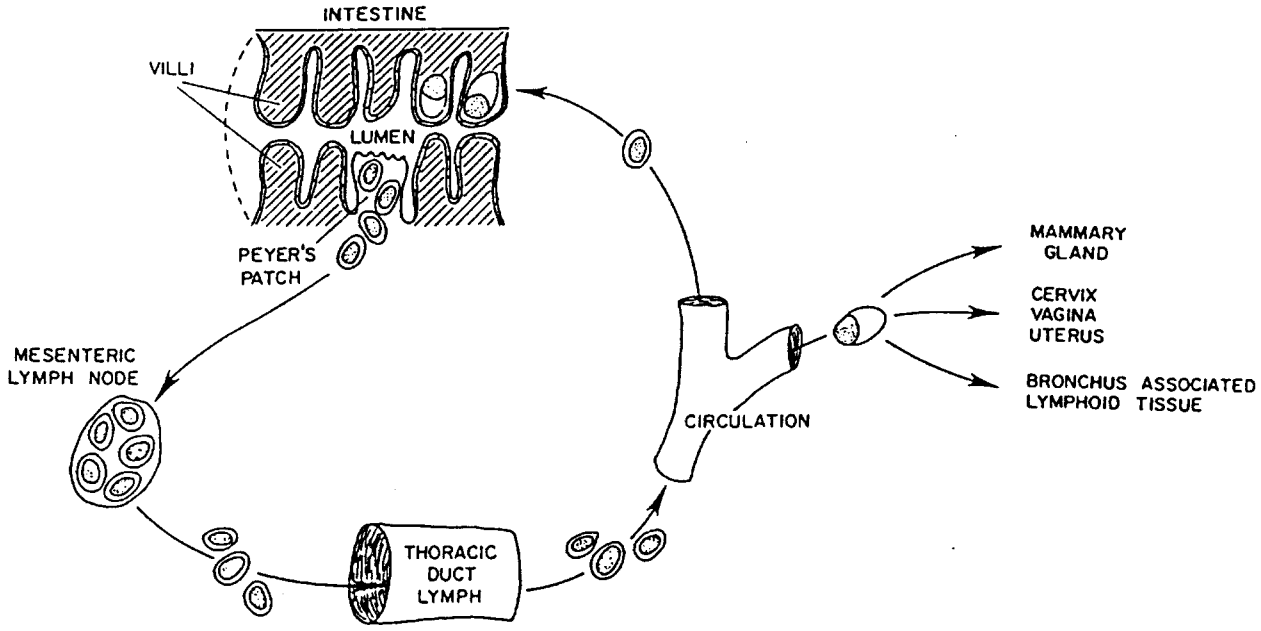


Figure: 1.6 Migration pathways of Peyer's patch lymphocytes (Kagnoff, 1987).

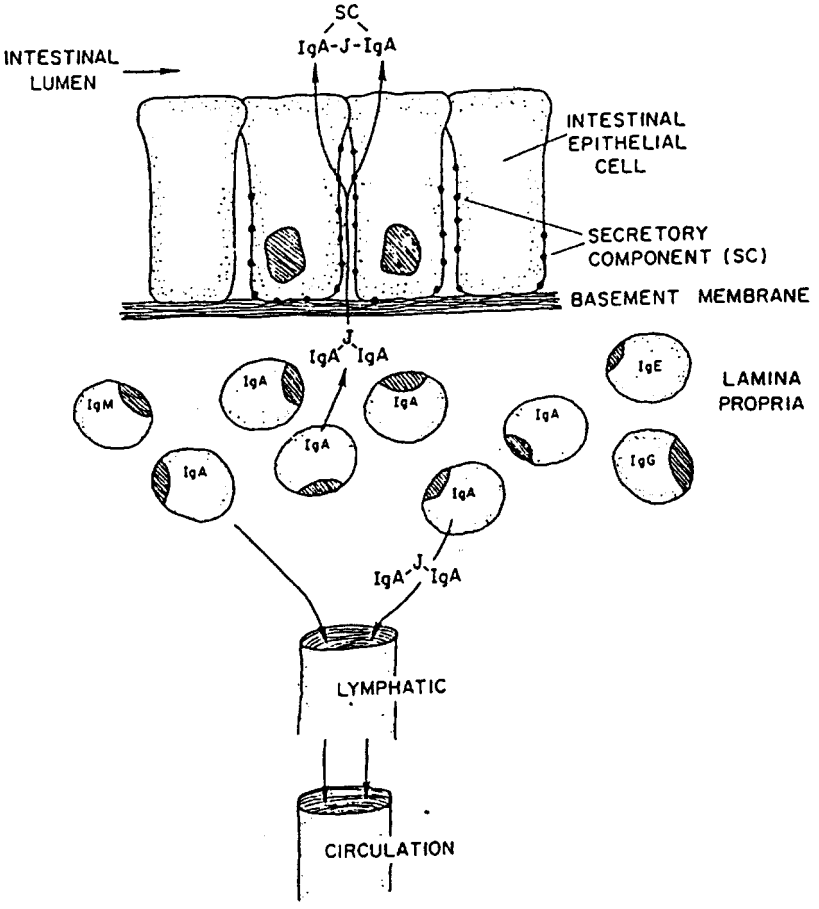


Figure: 1.7 Transport of IgA into intestinal secretions (Kagnoff, 1987).

IgA has been shown to have antibody activity to bacteria (Fubara and Freter,1973), virus (Riepenhoff-Talty et al.,1981) and other foreign particles (Walker et al.,1972). Secretory IgA prevents bacteria from attaching to and penetrating intestinal epithelial cells and thereby prevents colonization (Freter 1970; Williams and Gibbons,1972).

1.10. Background literature:

1.10.1. The prevalence of vitamin A deficiency in the world:

Surveys carried out by Moore (1957) in various countries in Europe, over a 20 year period (between 1930-1950) on post mortem liver samples have established the body reserves of vitamin A. These results showed that most people examined had adequate amounts of vitamin A. This view was changed with regard to the third world in the late 1960s, when numerous surveys revealed the extent of vitamin A deficiency particularly in the developing countries (Oomen et al.,1964; McLaren et al.,1965; Chopra and Kevany,1970; Varela et al.,1972). It has been claimed that after the deficiency of total energy and protein, vitamin A is the most common dietary deficiency in the world (Roels,1970). Bangladesh, India, Indonesia and Philippines appear to be the most affected but many parts of Africa and some in Central and South America have the same problem (Pirie,1983).

1.10.2. Vitamin A deficiency and diarrhoeal disease:

Several studies, linking vitamin A deficiency with increased risk of disease or death from diarrhoea, pay special attention to the relationship between vitamin A deficiency and diarrhoea among young children. Sommer et al (1984) in their observational studies, have shown an association between mild vitamin A deficiency and the risk of diarrhoea and respiratory disease. They have suggested that vitamin A deficiency, as expressed by mild xerophthalmia, predisposes to increased diarrhoeal incidence and increased mortality from all causes

among preschool aged children in West Java. Stoll et al (1985) have shown that diarrhoea patients (children under 1-10 years in Bangladesh) with night blindness were significantly more likely to have prolonged diarrhoea and dysenteric disease (specially by Shigella and Entamoeba histolytica) than patients without night blindness. Stanton et al (1986) have also shown an association between protracted diarrhoea and the prevalence of mild xerophthalmia in children under 14 years of age in slum areas in Dhaka, Bangladesh. From the above observations, it becomes evident that vitamin A deficiency aggravates infectious disease like diarrhoea, in addition to changes in the eye. However, in a recent review, Feachem (1987) discussed the intervention studies which controlled vitamin A deficiency and measured the impact on diarrhoea and concluded that the evidence which suggested vitamin A deficiency predisposes to increased risk of diarrhoea illness are limited and also suggested further studies are needed to establish the protective role of vitamin A supplementation on diarrhoeal illness.

1.10.3. Causes of diarrhoeal diseases:

Various types of bacteria and viruses may cause diarrhoeal diseases. W.H.O. Scientific Working Group (1980) have considered that viruses are an important cause of diarrhoeal episodes in infants and young children in both developed and underdeveloped countries. Among the viruses, rotavirus is one of the important causes of morbidity or mortality in humans (Kapikian et al.,1980) and animals (Snodgrass et al.,1976). The involvement of rotavirus in infantile gastrointestinal disease has been reported worldwide (Albert et al., 1983; Konno et al., 1978). In a recent report Su et al (1986) have described epidemic diarrhoea among adults in Anhui province of China in the summer of 1983, caused by rotavirus. Galil et al (1986) have also found rotavirus induced gastroenteritis in infants, children and adults in Southern Israel. In South Africa, about 23% of the gastroenteritis patients, those admitted in the Ga Rankuwa Hospital, were found positive for rotavirus infection (Steele et al.,1986). From all these

observations it is clear that worldwide rotavirus is one of the most important causes of gastroenteritis in both humans and animals.

1.10.4. Vitamin A deficient model:

Vitamin A status has been defined as follows: deficient, marginal, satisfactory, excessive and toxic. Clinical signs may be present in the deficient and toxic states, but not in the other three states. A satisfactory state is characterized by the absence of any signs of vitamin A deficiency and by the presence of an adequate body reserve of the vitamin A. A liver concentration of 20.0 µg/g is considered to provide an adequaté reserve (Olson,1987). When the body reserve of vitamin A is completely exhausted, the subjects are termed as vitamin A deficient.

Vitamin A deficiency results primarily from inadequate dietary intake of vitamin A. The usual method for producing a vitamin A deficient experimental animal model has been simply to feed normal weanling animals a vitamin A free diet until growth ceases (Moore, 1957). Most research workers have used weanling rats as a vitamin A deficient model to investigate vitamin A action (Nauss et al.,1985; Takagi and Nakano., 1983; Krishnan et al., 1976; Muto et al., 1972). In all these experiments, the workers have tried to use dietary ingredients which are thought to be vitamin A deficient or vitamin A free. In practice, the time required to make vitamin A deficient animals has varied in each of these experiments. Takagi and Nakano (1983) have shown their experimental animals begin to reduce their growth rate after 40 days on the dietary regimen. Others have found their animals entered the weight plateau stage after about 35 days of feeding the vitamin A deficient diet (Krishnan et al.,1976; Nauss et al., 1985). Muto et al (1972) have observed cessation of growth after 50 to 60 days on the diet. However, all these groups have demonstrated vitamin A deficiency in their rat model, either by cessation of weight gain alone or by both the weight gain and vitamin A levels in tissue or serum. Others have used hamsters or chicks as their experimental

vitamin A deficient model (Bonanni et al., 1973; Bang and Foard., 1971).

It is extremely difficult to produce a vitamin A deficient mouse model. McCarthey and Cerecedo (1952) have reported that mothers on a vitamin A deficient diet failed to produce vitamin A deficient weanlings or neonates, although the same diet regimen was able to produce vitamin A deficient rats. However they were able to produce vitamin A deficient mice by using a modified diet recipe, where vitamin A deficient symptoms appeared after 90-120 days of feeding the weanling mice. They have suggested that mice exhibit greater resistance with respect to body weight gain, life span and time of appearance of symptoms of vitamin A deficiency. Again a recent paper of Smith et al (1987) reported that feeding a vitamin A deficient diet to pregnant females during the 2nd week of gestation and continuing to feed their offspring the same diet produces vitamin A deficient mice at 8 weeks of age. Smith et al (1987) have suggested that rats and mice may differ with respect to vitamin A absorption, storage and metabolism. Therefore, extreme care is necessary to select the dietary ingredients for producing a suitable vitamin A deficient mouse model.

1.10.5. Vitamin A deficiency and infection:

Soon after experimental vitamin research began, a certain relation between the advanced stage of vitamin A deficiency and spontaneous infections was observed (Green and Mellanby,1928; Green and Mellanby,1930; Turner et al.,1930). In the rats the most frequent spontaneous infections were: abscesses at the base of the tongue, xerophthalmia, infection of the urinary tract (Green and Mellanby,1928) and infection of the upper respiratory tract (Turner et al.,1930). On the other hand, several others have shown the course of experimental infections in animals on vitamin A deficient diet. For instance, Werkman (1923) showed that vitamin A deficient rats had lowered resistance against subcutaneous injection of typhoid bacilli and intraperitoneal injection of anthrax bacilli compared with control

animals. Lassen (1930) has also shown a lowered resistance in vitamin A deficient rats infected with a paratyphoid bacilli compared with controls. Further, the course of infection and bacteriological findings were the same whether the organisms were inoculated by mouth or subcutaneously. Boynton and Bradford (1931) have demonstrated that vitamin A deficient rats had a markedly decreased resistance to intraperitoneal inoculation with an encapsulated bacillus of the mucosus group compared with controls and that this susceptibility was demonstrable before any other symptoms of vitamin A deficiency. A marked increase in susceptibility was observed when Salmonella enteritidis was injected intraperitoneally in a group of animals fed a vitamin A deficient diet for a period of seven weeks compared with controls (McClung and Winters, 1932). Greene (1933) has shown that vitamin A deficient rabbits were more susceptible to intranasal infection with Bact.lepisepticum and intravenous infection with type I pneumococcus than control rats.

In contrast, Cramer and Kingsbury (1924) have reported that inoculation with different bacteria (B.anthrasis, Streptococci, B.Coli) did not reveal any obvious diminuation in the general resistance of the vitamin A deficient rats. The only exception they found was in infections with B.tuberculosis (bovine type), where vitamin A deficient animals had enlarged mesenteric nodes which gave positive smear tests after 4 weeks of intraperitoneal challenge. The control animals received the same injection but the glands were not enlarged. Clapham (1933) has also shown that vitamin A deficiency in chickens had no effect on the resistance to infestation with Heterakis gallinae. However the same author showed that when vitamin A deficient rats were infected with Parascaris equorum, there was a greater survival and increased rate of larval development of P.equorum than in controls. Lawler (1941) has shown that partial vitamin A deficiency in rats did not have any effect on the resistance to subcutaneous infection with Strongyloides ratti. This author also showed that when liver vitamin A reserves were completely exhausted the rats had a lower resistance to Strongyloides ratti than controls.

Kligler et al (1945) have shown that vitamin A deficient mice and rats, when infected orally with Salmonella typhimurium, were more susceptible than normally fed controls as judged by mortality and the number of infective organisms observed in liver, spleen and mesenteric nodes. These authors using pairfed animals in a separate experiment showed that the pairfed animals were equally susceptible to this enteric bacterial infection as vitamin A deficient animals. Therefore they have suggested that low food intake rather than vitamin A deficiency was responsible for the lowered resistance to Salmonella typhimurium.

Sriramachari and Gopalan (1958) have investigated the effects of deficiencies of protein, vitamin A and calories on resistance to tuberculosis in rats. About 60-65 % of the animals from all the groups except the vitamin A deficient group were found to be infected, whereas in the vitamin A deficient group 81 % of the animals became infected. They measured the extent and severity of infection in the animals receiving 2, 5, and 18 % protein diets and found the same degree of involvement in all three groups. The results were similar in the group receiving high protein but low calorie diet , whereas vitamin A deficient animals exhibited relatively more severe infection. These workers did not see any appreciable influence of the level of protein, or 50 % restriction of calorie intake on the resistance of rats to tuberculosis infection or the mortality. However, they point out that these studies only dealt with the immediate or short term effect of infection. Under the same conditions, the incidence of infection was considerably higher and the lesions also more extensive in the vitamin A deficient group compared with controls.

In vitamin A deficiency, experimental ocular Herpes simplex virus (HSV) infections were found to be more severe in rats compared with the normal pairfed control (Nauss et al., 1985a) and resulted in a higher incidence of epithelial ulceration and necrosis. Rogers et al (1971) have shown that the vitamin A deficient rats survive for longer periods when kept in a germ free state, whereas early death is

observed in a conventional environment. Their observations have suggested the importance of vitamin A in protecting against infection. Decreased resistance to infection with Newcastle disease virus (NDV) has been reported in vitamin A deficient chicks where approximately 100 times more virus was found in the throat swabs from the deficient than in the normal chicks after intranasal inoculation with the virus (Bang and Foard., 1971). This group also showed the increased susceptibility of vitamin A deficient chicks to influenza virus.

The ability of vitamin A deficient rats to resist infection with the malarial parasite Plasmodium Berghei was investigated by Krishnan et al (1976). They have shown that the parasitaemia increased at a faster rate in the vitamin A deficient group than the controls. They did not see any significant difference in parasitaemia between the control and pairfed groups. Further, vitamin A deficient animals died within 6-7 days of infection, whereas the survival time of pairfed animals was 9-11 days . None of the infected ad libitum control group died during the 5 weeks observation period. They have also shown that the oral supplement of retinyl acetate to vitamin A deficient rats enabled the animals to recover from the infection. The same group also examined the effect of a partial state of vitamin A deficiency on resistance to a subclinical infection. The parasitaemia increased markedly in the rats fed the deficient diet after 10 days of infection and animals died on day 30 after infection. None of the pairfed or control series died during the 5 week period of observation.

Vitamin A deficiency leads to an increased susceptibility to infection by the rat lung worm, Angiostrongylus cantonensis after subcutaneous challenge (Darip et al., 1979). This group have shown that after the infection, all the vitamin A deficient animals died with an average survival time of only 39 days, whereas controls remained alive and healthy throughout the observation period (60 days). Further, the number of worms recovered from the pulmonary arteries and lungs of vitamin A deficient animals was significantly higher than the control animals. They have further demonstrated by

oral challenge that more larvae were able to penetrate the intestinal mucosa of vitamin A deficient rats than controls and suggested that vitamin A is an important regulating factor at the site of entry, but when the parasites have invaded the tissue, they are able to migrate and develop normally, at least during the early phase of worm developement, regardless of the vitamin A status. Parent et al (1984) have shown that the vitamin A deficient rats when infected with a parasite, Schistosoma mansoni, had a larger adult worm population as compared to controls. They have also shown that the liver of vitamin A deficient animals contained many eggs of S.mansoni, whereas in the control liver no eggs were found.

Vitamin A supplementation has been shown to enhance resistance against some bacterial and fungal organisms. Adler and Damassa (1972) have demonstrated that normal adult birds (Coturnix Quail) escaped the pathologic effect of Arizona hinshawii bacteria when vitamin A acetate was administered concomitantly with the bacterial inoculum. A similar effect has been observed in mice using vitamin A against other bacterial species and fungi (Cohen and Elin.,1974).

1.10.6. Immunopotentiating effect of vitamin A:

The immunological adjuvant properties of vitamin A have been described repeatedly. Vitamin A causes an increased antibody response against dinitrophenylated ovalbumin (DNP-OVA) in mice (Cohen and Cohen,1973) and vitamin A treatment also enhances the number of antibody forming cells in the spleen in response to sheep red blood cells (SRBC). A single dose of 150 µg vitamin A palmitate also enhances the cell mediated immunity to sheep red blood cells as early as 3 days following immunization (Athanassiades,1981). Barnett and Bryant (1980) have shown that retinol is an immunopotentiating agent and acts as an adjuvant for the IgG ovalbumin response in mice. This same group also showed enhanced IgE production against ovalbumin in mice fed a retinol supplemented diet (Bryant and Barnett, 1979). Jurin

and Tannock (1972) have shown that vitamin A acts as an adjuvant for the sheep RBC response in mice and can stimulate both humoral and cell mediated immunity. Vitamin A also enhances the response to oral immunization as it increases both the local and systemic antibody response following Bovine serum albumin administration in female Swiss albino mice (Falchuk et al.,1977). Vitamin A alcohol has also been shown by Dresser (1968) to posses extrinsic adjuvanticity, increasing antibody formation against bovine γ-globulin. Floersheim and Bollag (1972) have further demonstrated that vitamin A acid, an analog of retinol, can accelerate the rejection of skin homografts in mice indicating that it is also effective in cell mediated immunity. Salti and Murad (1985) have shown that vitamin A in a nontoxic dose may have a role in enhancing spleen lymphocyte transformation to weak immunogens. They have also shown that the anti tetanus and anti BCG titre was significantly higher in the vitamin A treated animals than the control group. In addition to the adjuvant effect of vitamin A, it also has an anti immunosuppressive effect. Vitamin A prevents the immunosuppressive effects, both humoral and cellular, of prednisolone and cyclophosphamide in mice (Salti and Murad,1985). Cohen and Cohen (1973) have also demonstrated that the simultaneous administration of vitamin A can prevent the immunosuppressive effect of hydrocortisone on the response to sheep RBC.

The immunopotentiating effect of vitamin A or retinoids has been demonstrated in many other experiments. For instance, Malkovsky et al (1983) have shown that a vitamin A acetate rich (0.5 gm/kg diet) diet fed mice immunized with irradiated cloned fibrosarcoma cells displayed substantially enhanced tumor specific immunity in vivo when challenged with viable tumor cells of the immunizing cell line, as compared to control mice. This enhanced tumor directed immunity was demonstrated in that specific immunization markedly decreased the incidence of tumors after the McSa-1 tumor cell challenge in a group of mice fed vitamin A acetate diet (5% tumor) as compared to controls (50% tumor). Survival time of the immunized mice on the vitamin A acetate rich diet was also significantly prolonged in comparison with

a control group of mice following challenge with viable sarcoma cells.

Malkovsky et al (1984) have shown that increased resistance to transplanted tumor in vivo in mice was linearly dependent on the dose of vitamin A acetate supplementation to an otherwise conventional diet and also that there was a positive linear relationship between the resistance to transplanted tumor and the length of exposure to supplementary vitamin A acetate relative to tumor inoculum time. Colizzi and Malkovsky (1985) have shown that high dose mycobacterium bovis infected mice fed a vitamin A acetate-supplemented diet developed a positive skin reaction to purified protein derivative of mycobacteria whereas a very poor response was observed in M.bovis BCG infected mice on a conventional diet. They have suggested that the functional activity of interleukin-2 secreting T cells and delayed type hypersensitivity mediating T cells were significantly influenced both by BCG and vitamin A acetate. Malkovsky et al (1983a) have reported that mice fed vitamin A acetate supplemented diet responded to semiallogenic cells in a host-versus-graft (HvG) reaction, whereas mice on a conventional diet did not, and further that vitamin A acetate fed mice had a significantly higher proportion of T cells expressing Lyt 1.1 cell surface antigen in their lymph nodes than control mice and suggesting that the increased immune responsiveness is due to the Lyt 1.1 phenotype positive T cell population.

Kazt et al (1987) have investigated the mechanism which is responsible for the immunomodulatory activities of retinoids and suggested that the number of accessory cells present in the lymphomedullary tissue was increased by vitamin A acetate supplemented diet and the function of these cells was increased. Further, the effect was more pronounced for vitamin A acetate than 13-cis-retinoic acid. Vitamin A acetate has also been shown to stimulate cell mediated immunity in that mice fed on a vitamin A acetate supplemented diet showed enchanced delayed hypersensitivity to skin contact sensitizer, oxazolone, compared with mice fed a standard diet (Miller et al., 1984). This group have shown that application of relatively high dose of oxazolone to vitamin A acetate fed mice compared with mice on

standard diet resulted in a significant increase in DNA synthesis in the lymph nodes and a significant increase in ear thickness after elicitation. They have suggested that vitamin A acetate enhances all T-T cell interactions including those involved in the delayed hypersensitivity reaction.

1.10.7. Vitamin A deficiency and immune response:

1.10.7.1. Lymphoid Tissue:

Marked atrophy of the thymus and spleen is claimed in vitamin A deficient rats in comparison with pairfed and control rats, resulting in reduced immune responsiveness (Krishnan et al.,1974). These authors have shown that the thymic cortex of vitamin A deficient animals was almost completely depleted of lymphocytes. However, they have also shown cortical depletion of lymphoid cells in the pairfed control rats but to a less pronounced level. Similarly they observed atrophic changes in the spleen where involution of the germinal centres occured. They have suggested that the changes seen are mostly due to concomitant protein-calorie undernutrition.

Nauss et al (1979) have shown no significant changes in either relative thymus weights or relative spleen weights of vitamin A deficient animals compared with control or pairfed animals. However the total spleen weight and total thymus weight of vitamin A deficient animals were significantly lower than those of ad libitum control animals. Again total thymus weight but not the spleen weight was significantly lower than the pairfed group. This result indicates that the thymus is affected by vitamin A deficiency. This group have also shown that the total liver weight and relative liver weight of vitamin A deficient animals were significantly lower than the control but not the pairfed group. Nauss and Newberne (1985) have shown low spleen weight, whether expressed as total weight or weight per 100 gram of body weight, in vitamin A deficient animals compared with pairfed controls. In contrast, the cervical lymph nodes of vitamin A deficient

animals weighed significantly more than those of their pairfed controls, and a further weight increase was seen in response to Herpes simplex virus infection.

Nauss et al (1985) have investigated the progressive effect of vitamin A deficiency on the immune response and shown that the relative spleen weights of vitamin A deficient animals were not significantly different from the pairfed group either in the early or late stages of vitamin A deficiency. The weight of the cervical and mesenteric lymph nodes was greater during the late stage of vitamin A deficiency compared with the pairfed group. They have also shown that the splenic lymphocyte yields (per mg tissue) did not differ between the early stage of vitamin A deficiency and pairfed controls but that there was a significant difference in the later stage. Although there was a progressive increase in weight of the mesenteric lymph node of vitamin A deficient rats compared with pairfed animals, there was no significant difference observed in lymphocyte yield per mg tissue. They have also shown that the yield of viable lymphocytes from cervical lymph node in vitamin A deficient animals were similar to control except in the late stages of vitamin A deficiency, when lymphocyte yields were higher than those of the pairfed control group. The above result indicate that lymphoid organ weights do not necessarily reflect the number of lymphocytes present in the organ. The increased weight of the lymph nodes was explained by the possibility of subclinical infection in the deficient animals.

Takagi and Nakano (1983) have failed to demonstrate a significant difference between non-immunized spleen weights of either vitamin A deficient or control animals. However, spleen weights of SRBC-immunized, vitamin A deficient animals were significantly lower compared with controls. Others have shown the effect of vitamin A deficiency on organ weight in chicks. Davis and Sell (1983), have shown that vitamin A deficient broiler chicks show decreased relative thymus and bursa weights compared with any other dietary group fed retinol or retinyl acetate. They have suggested that the reduction of relative thymus or bursa weight is not entirely due to undernutrition

but the direct result of vitamin A deficiency. Panda and Combs (1963) have also shown that the relative bursal weights of vitamin A deficient chicks were significantly lower than controls.

Recent work of Smith et al (1987) has not demonstrated a significant difference in spleen, thymus or liver weight of vitamin A deficient mice compared with control mice. Although this group performed experiments at an early stage of vitamin A deficiency (before the body weight plateau phase), the serum vitamin A level was <20 % of the control value. They have also reported no gross abnormalities in total nucleated cells/organ in vitamin A deficient mice. However, in the later stage of vitamin A deficiency, when the serum vitamin A level was <10 % of the control value, higher spleen and lymph node weights were found. Further, the splenomegaly of vitamin A deficient mice was found to be more pronounced in the pathogen free than in the pathogen exposed animals. A small but significant increase in both the proportion and the absolute number of B-lymphocytes in spleen and lymph node was responsible for the increased organ weight gain with vitamin A deficiency and inanition. The T cell population remained unchanged in the thymus, spleen and lymph node. These authors suggest that the enlargement of lymphoid organs is not the direct consequence of vitamin A deficiency but the combined effect of vitamin A deficiency and inanition.

1.10.7.2. Cell mediated immunity:

The effect of vitamin A deficiency on the cell mediated immunity was examined by in vitro studies by many workers in this field. For instance, Nauss et al (1979) have shown that the splenic lymphocytes from deficient rats had one third the transformation response to the mitogens Concanavalin A (Con A), Phytohemagglutinin (PHA) and E.Coli lipopolysaccharide S (LPS) compared with control and pairfed groups. They have demonstrated that the in vitro transformation of lymphocytes with the use of tritiated thymidine in the culture system and observed that the control groups incorporated

more tritiated thymidine than the deficient animals. There was no significant difference observed between control and pairfed groups. Again these workers did not see any significant difference in thymus lymphocyte transformation in response to Con A, as a result of vitamin A deficiency. Further, when the vitamin A deficient rats were supplemented with Vitamin A, the transformation response returned to control values within 3 days, suggesting that vitamin A deficiency could result in a metabolic defect in the synthesis of the lymphocyte membrane receptors leading to an altered mitogen receptor site. This data raises the possibility that lymphocytes from vitamin A deficient animals are defective in the synthesis of glycoprotein which results in impaired mitogen binding and loss of proliferative responses. Nauss and Newberne (1985) have shown that the Concanavalin A induced splenic transformation response of non-infected vitamin A deficient rats was only 13% that of non-infected pairfed controls. However there was marked decrease in the splenic response to Con A in both dietary groups 3 days after Herpes simplex virus infection which remained up to 10 days of the experimental period. Again at all points the response from vitamin A deficient animals was significantly lower than the response from control animals. The cervical lymph node transformation response to Con A was higher in deficient animals than control animals. Further splenic natural killer cytotoxic responses were higher in control rats than vitamin A deficient animals and decreased in both groups during the 10 day post infection period.

Nauss et al (1985) have shown that a significant depression in the Con A induced transformation response of splenocytes from vitamin A deficient rats occur prior to the weight plateau stage (early stage of vitamin A deficiency). The reduction in both the Con A and Pokeweed mitogen (PWM)induced splenic responses was most marked during the first 2-5 days of the weight plateau. In contrast, the regional lymph nodes response of vitamin A deficient animals was normal during the early stage and became significantly different (elevated) as the deficiency progressed. This suggests that the lymphocyte transformation response depends on the stage of vitamin A

deficiency as well as the lymphoid organ being examined. In vitamin A deficient chicks, there is an inverse relationship between the number of peripheral blood lymphocytes obtained per ml of blood and their ability to proliferate in response to mitogenic stimulation (Davis and Sell, 1983). Peripheral blood lymphocytes of severely vitamin A deficient chicks exhibit strong proliferation in responses to mitogenic stimulation with Con A. This enhanced response was accompanied by a decrease in the number of lymphocytes per ml of blood.

Smith et al (1987) have demonstrated the effect of vitamin A deficiency on the cell mediated immunity in vivo by using the delayed-type hypersensitivity response. This group have shown that vitamin A deficient mice exhibit a significantly lower DTH response than control mice. The response was lower either at a very early (serum vitamin A level was <50 %) or late stages of vitamin A deficiency.

1.10.7.3. Humoral Immunity:

The effect of vitamin A deficiency on antibody production has been examined in several studies. For instance, Werkman (1923) has shown that rabbits and rats on vitamin A deficient diets showed no differences in their agglutinin and bacteriolytic responses to immunization with B.typhosus compared with that of control animals. Cramer and Kingsbury (1924) have also studied the production of typhoid agglutinin in rats on vitamin A free diet injected B.typhosus intraperitoneally and found to be normal when compared with controls. In contrast, Greene (1933) has shown a marked lack of anti-sheep or ox hemolysin titre in vitamin A deficient rabbits after intravenous immunization compared with that of controls. This author also claimed a tendency for a vitamin A deficient rabbits to show lower antibody titres than controls following intravenous immunization with B.typhosus. However, many of these studies were carried without the benefit of purified diets. Ludovici and Axelrod (1951) observed that

rats fed a vitamin A deficient diet for 4 weeks had lower hemagglutination titres in response to intraperitoneal injection of human erythrocytes when compared with controls. Pruzansky and Axelrod (1955) have shown a similar effect on antibody production against diphtheria toxoid injected intraperitoneally in vitamin A deficient rats. Chicks fed diets partially deficient in either vitamin A , pantothenic acid or riboflavin showed a significantly lower agglutinin response to S. Pullorum antigen compared with controls (Panda and Combs, 1963). Krishnan et al (1974) have shown that vitamin A deficient rats had significantly lower antibody titre against diphtheria and tetanus toxoids compared with control or pairfed animals. However, there was no significant difference in antibody titre raised against SRBC between pairfed and vitamin A deficient animals. Since all these antigens were used under the same experimental protocol, it is possible that the effect of vitamin A deficiency on the antibody levels depends on the type of antigen. Again Davis and Sell (1983) have shown that neither a total nor a partial deficiency of dietary retinol or retinyl acetate adversely affected the antibody titres of broiler chicks in response to human serum albumin. Leutskaya and Fais (1977) have shown that circulating antibody levels and in vitro antibody production in isolated spleen cells from presensitized chicks were lowered by vitamin A deficiency. The addition of retinyl palmitate to the incubation medium reversed this effect.

Moderate vitamin A deficiency (serum vitamin A level was <50 % of the control) did not have any effect on the antibody production (IgM antibody response) against a single dose of intraperitoneal hemocyanin (Smith et al., 1987), whilst a significantly low level of IgM antibody is seen in vitamin A deficient animals (serum vitamin A level was <20 % of the control) compared with controls. Smith et al (1987) suggest that impaired humoral immunity is a direct consequence of vitamin A deficiency, because at this point both vitamin A deficient and control animals had eaten the same amount of diet. Further, the kinetics of antibody production remained unchanged due to

the vitamin A deficiency. There was an increase in both the proportion and the absolute number of B-lymphocytes in lymph node and spleen of vitamin A deficient animals, despite their low levels of IgM antibody against hemocyanin. These authors feel that vitamin A deficiency leads to a defective rather than a depleted cell population leading to the reduced antibody levels. Sirisinha et al (1980) have investigated the effect of vitamin A deficiency on the production of different subclases of immunoglobulin and have shown that the secretory IgA levels in the intestinal fluid of vitamin A deficient rats were significantly lower than controls. However, IgG levels in the intestinal fluid, and serum IgA and IgG levels were unaffected in deficiency. This group have also shown that whilst the local anti -DNP responses were depressed in vitamin A deficient rats as compared to controls the systemic anti-DNP responses were only affected marginally by vitamin A deficiency. They suggest that vitamin A deficiency does not affect the production of IgA by the plasma cells but the synthesis of secretory component is adversly affected, making it more difficult for the IgA dimer to pass into the intestinal lumen.

Other reports on the immunological effects of vitamin A supplementation are more controversial. Ways et al (1980) found no significant change in immunocompetence (antibody titre and cell mediated immunity) in neonatally vitamin A treated animals and Brown et al (1980) found no difference between vitamin A treated or control children in the tetanus antitoxin responses following tetanus toxoid immunization or in skin test reactivity to common antigens. Further, Underdahl and Young (1956) claim no effect of vitamin A on the formation of antibodies to influenza virus in mice.

1.10.8. Conclusion from the literature on vitamin A and immunity:

In 1920s several studies have shown that experimental vitamin A deficient animals were more susceptible to the spontaneous infections (Green and Mellanby,1928; Turner et al., 1930). During 1920-1940s several others have shown that vitamin A deficient animals

were more susceptible to experimental bacterial (Werkman,1923; Lassen,1930; Boynton and Bradford,1931; Greene,1933) and parasitic (Lawler,1941) infections as compared to that of controls. In contrast, others did not see any difference on the resistance to these infections due to vitamin A deficiency (Cramer and Kingsbury,1924; Clapham,1933). However, most of these studies were carried out on a non-purifed diet. In recent years many workers have shown that vitamin A deficient animals responded poorly when challenged with infectious diseases (Krishnan, 1976; Darip et al, 1979; Parent et al., 1984). This impaired resistance to infectious disease could not be explained by changes in the lymphoid organ weights, because the weights of these organ were varied in different studies (Nauss et al, 1979; Nauss and Newberne, 1985; Smith et al, 1987). Again the effect of vitamin A deficiency differs from organ to organ (Nauss et al, 1985), where they have shown no effect on the spleen weights due to vitamin A deficiency but the weight of the mesenteric lymph nodes were higher compared with pairfed group.

Many studies have shown that the supplementation with vitamin A can enhance immune responses (Malkovsky et al., 1983a; Colizzi and Malkovsky, 1985; Miller et al., 1984; Floersheim and Bollag, 1972). All these studies have shown increased cell mediated immunity in the vitamin A or retinoid supplemented animals as compared to controls. Others have shown increased antibody levels in retinol supplemented animals compared with controls (Barnett and Bryant., 1980; Dresser, 1968; Jurin and Tannock., 1972; Bryant and Barnett,1979). Falchuk et al (1977) have also shown that retinol supplementation can enhance both the local and systemic immunity.

On the other hand, vitamin A deficiency is associated with depressed cell-mediated immunity either in vitro (Nauss et al., 1979; Nauss et al., 1985) or in vivo (Smith et al., 1987). Others have failed to show any alteration in the cell mediated immunity due to vitamin A deficiency (Parent et al., 1984). Similarly, whilst vitamin A deficient animals have been shown to exhibit poor antibody responses to different antigenic challenges by some workers (Ludovici and

Axelrod,1951; Krishnan et al., 1974; Panda and Combs, 1963; Leutskaya and Fais, 1977; Smith et al., 1987), others have claimed that there is no significant change in the antibody responses in either viamin A treated animals (Ways et al, 1980) or vitamin A deficient animals (Werkman,1923; Underdahl and Young, 1956; Davis and Sell, 1983).

In man, retinoids enhance antibody responses as shown by increased numbers of antibody plaque forming cells (Sidell et al., 1984), whilst Brown et al (1980) did not see any difference of antibody response between vitamin A deficient and control children following tetanus toxoid immunization.

The relationship between vitamin A deficiency and the immune response is not altogether clear, however many studies have shown poor resistance to infectious agents.

1.11. Objective of the study:

Vitamin A deficiency is one of the major nutritional problems throughout the world, specially in developing countries. As already reviewed in this chapter, many studies have shown that vitamin A has a wide range of physiological and biochemical actions. The relationship between vitamin A and the immune response is however far from clear. Several reports have indicated that vitamin A causes an immunopotentiation and acts as an adjuvant with a variety of antigens (Jurin and Tannock,1972; Cohen and Cohen,1973; Barnett and Bryant,1980; Dresser,1968) and vitamin A deficient subjects show poor antibody responses to different antigenic challenges (Krishnan et al.,1974; Leutskaya and Fais,1977; Smith et al., 1987). Other authors have claimed that there is no significant change in antibody responses in vitamin A treated animals (Ways et al.,1980; Underdahl and young,1956) or children (Brown et al 1980).

Rotavirus represents one of the major causes of gastroenteritis in both developed and underdeveloped countries and acts via the epithelium of the intestine. Since one of the most important functions of vitamin A is the maintenance of the epithelia

of various organs (Wolbech, 1954), we considered it of interest to investigate the interaction between vitamin A and rotavirus infection.

Clearly it is not possible to carry out a definitive study in humans to demonstrate that a vitamin A deficient individual is more susceptible to an infectious disease. It is much easier to investigate these problems in an animal model.

Human rotaviruses are morphologically similar to some animal rotaviruses specially the agent causing epizootic diarrhoea in infant mice (EDIM). The EDIM strain of rotavirus and the pathology of the disease in mice resembles closely diarrhoea caused by rotavirus in humans (Bishop et al., 1973; Cheever and Mueller, 1947).

These observations prompted us to study the effect of vitamin A deficiency on the immune response against murine rotavirus infection in mice .

With this end in view, the following parameters were investigated:-

1) Weight gain and growth of different lymphoid organs especially spleen and thymus.
2) Gut histology.
3) Histology of the lymphoid organs.
4) Liver and serum vitamin A estimation.
5) Non specific immunity, i.e. mucin status.
6) Levels of circulating total antibody specific to rotavirus.
7) Delayed hypersensitivity response as a measure of cell mediated immunity.

CHAPTER TWO

MATERIALS AND METHODS

CHAPTER TWO

Materials and methods

This chapter describes in detail the methods employed in the studies which are reported in the following chapters.

2.1. Materials:

The strain of rotavirus preparation which produces Epizootic diarrhoea of infant mouse (EDIM) was kindly provided by the Microbiology Department, Birmingham University. The same strain was used in all the experiments.

2.2. Methods:

The animals used in this study were obtained from the Southampton University Medical School colony. Weanling male Porton mice were used for all the experiments. Animals were divided into three groups. The first group, designated the deficient group was fed a vitamin A deficient diet as shown in the table: 2.1, which supplied all other nutrients at the level recommended for these animals (Am.Inst.Nutr. 1977). Two control groups, one fed ad libitum and the other pairfed to the deficient animals, received the same diet and in addition 4000 I.U. vitamin A (stabilized powder, Roche)/kg diet.

All animals had free access to food and water, except the animals in the pairfed group. Pairfed mice were offered the same weight of food as the deficient mice had eaten on the previous day.

Initially the animals of all groups were approximately weight matched. During experiments the body weights were measured weekly and food consumption was measured daily.

Table: 2.1

Composition of the vitamin A deficient diet (modification of AIN-76)

Ingradient	Percentage(g/100g)
Vitamin free casein[1]	20.0
Corn starch[2]	45.5
Sucrose	20.0
Cellulose	5.0
Corn oil[3]	5.0
Vitamin Mix[4]	1.0
Mineral Mix[5]	3.5

1. Obtained from Special Diet Services, U.K.
2. Obtained from CPC product, U.K.
3. Obtained from London oil medina, London.

4 & 5 Compositions are shown in table: 2.2 and 2.3 respectively.

Table: 2.2

AIN-76 modified vitamin mixture (Am.Inst.Nutr. 1977).

Vitamin	mixture(mg/kg)
Thiamin-HCl	600.0
Riboflavin	600.0
Pyridoxine	700.0
Nicotinic acid	3000.0
Calcium pentothenate	1600.0
Folic acid	200.0
Biotin	20.0
Cyanocobalamin	1.0
Vitamin D (100,000 IU)	200.0
Vitamin E (5000 IU)	10,000.0
Vitamin K	5.0
Inositol	10,000.0
Choline	10,000.0
Sucrose to make	1000.0 g

Table: 2.3

AIN-76 modified mineral mixture (Am.Inst.Nutr. 1977).

Ingradient	Mixture (g/kg)
Calcium phosphate, dibasic	500.0
Sodium chloride	74.0
Potassium chloride	42.0
Potassium sulphate	52.0
Magnesium hydroxide	35.0
Manganous carbonate	3.5
Ferric citrate	6.0
Zinc carbonate	1.6
Cupric carbonate	0.3
Potassium iodate	0.01
Sodium selenate	0.01
Chromium potassium sulphate	0.55
Sodium molybdate	0.01
Sucrose to make	1000.0 g

Animals were housed in groups of 2-3 in each cage. The room temperature was maintained at 22±2°C and 50 % humidity. The rooms provided 14 hours light and 10 hours of darkness.

2.3. Estimation of liver vitamin A :

Liver vitamin A was determined by using a direct solvent extraction method of Bayfield (1975) with modifications.

2.3.1. Reagents:

(a) Ethanol.
(b) Hexane.
(c) Redistilled chloroform.
(d) Trichloro acetic acid (TCA) solution: 50.0 g of TCA were dissolved in 25.0 ml of redistilled chloroform. This reagent was always freshly prepared.
(e) Vitamin A acetate solution (stock solution): 10.0 mg of vitamin A acetate was dissolved in 10.0 ml chloroform.

2.3.2. Standard curve:

1.0 ml stock solution of vitamin A acetate was diluted in 50.0 ml of chloroform to give a concentration of 20.0 µg/ml. Appropriate aliquots of this diluted vitamin A acetate were taken to prepare concentrations of 2.0, 4.0, 6.0, and 8.0 µg/ml and sufficient chloroform was added to make 1.0 ml followed by 2.0 ml TCA reagent. Optical density was measured at 620 nm. The control blank contained 1.0 ml chloroform and 2.0 ml TCA solution. The standard curve for vitamin A acetate is shown in figure: 2.1.

2.3.3. Extraction of vitamin A:

About 1.0 g of liver was weighed into a homogenizing tube and

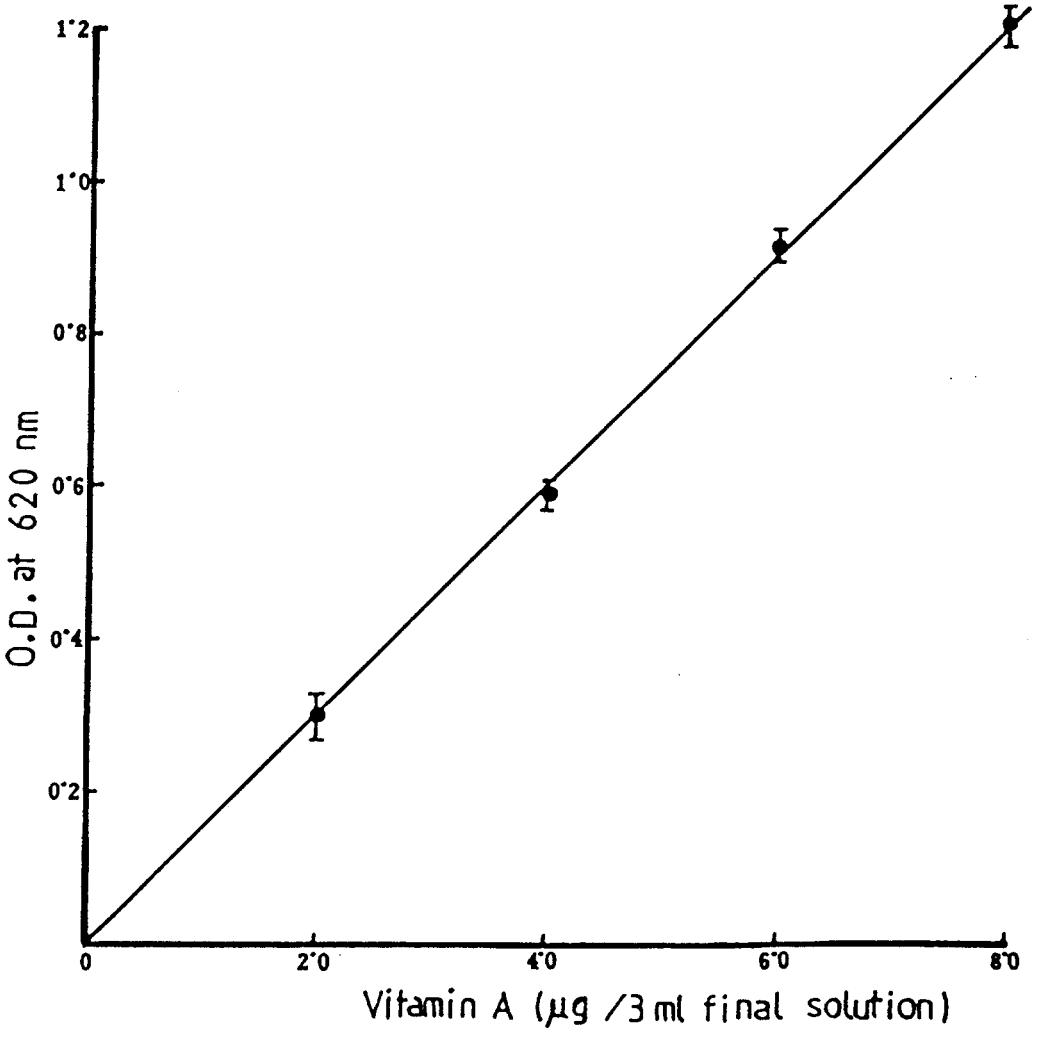


Figure: 2.1. Standard curve for liver vitamin A. Each point indicates the mean of three readings±S.E.

homogenized in ethanol. Hexane was added in a ratio of 1:2.5 ethanol:hexane. The solutions were mixed in a vortex mixer for 1 minute, followed by centrifugation for 10 minutes at 1000 X g. Vitamin A was extracted into the top hexane layer, which was collected and evaporated to dryness using a rotavapour apparatus at 40°C.

2.3.4. Determination of vitamin A:

The vitamin A extract was redissolved in chloroform and an appropriate aliquot was further diluted in chloroform to make 1.0 ml final volume in the reaction tube. The tube was placed in the spectrophotometer and 2.0 ml TCA reagent was added. The optical density was measured at 620 nm. The concentration was read from the calibration curve prepared by serial dilution of the standard.

2.4. Estimation of serum vitamin A:

2.4.1. Spectrophotometric method:

Serum vitamin A was measured in the form of retinol by the micromethod of Bradley and Hornbeck (1973) with slight modifications.

2.4.1.1. Reagents:

(a) Ethanol.
(b) Hexane.
(c) Redistilled choloroform.
(d) Tri fluro acetic acid (TFA).

2.4.1.2. Standard curve for β-carotene:

A stock solution of β-carotene (200.0 µg/ml) was prepared in hexane. This was then diluted to give solutions of 50.0, 100.0, 150.0, and 200.0 µg/100 ml hexane. 0.35 ml of each concentration was taken in

a microcell and the optical density at 450 nm was read against a hexane blank. The standard curve for β-carotene is shown in figure: 2.2.

2.4.1.3. Standard curve for vitamin A acetate:

A stock solution of vitamin A acetate (200.0 µg/ml) was prepared in chloroform. This was diluted further in chloroform to prepare standard solutions of 25.0, 50.0, 75.0, and 100.0 µg/100 ml. 0.2 ml of each solution was taken in a microcell and placed in the spectrophotometer: 0.1 ml TFA was added, mixed well and the optical density was read at 620 nm 15 seconds after the addition of TFA. The standard curve is shown in figure: 2.3.

2.4.1.4. Extraction and measurement of serum vitamin A:

0.2 ml serum samples were added to 0.2 ml of ethanol and 0.5 ml of hexane in an eppendorf tube. The solvent and sample were then mixed in a vortex mixer for 1 minute and centrifuged for 5 minutes in a microcentrifuge at 2000 X g. The top hexane layer was then collected. From this, an aliquot of 0.35 ml was pipetted and the carotene content was measured at 450 nm using a microcell.

The microcell contents were then poured into a small tube, the microcell rinsed once with hexane and the wash solvent added to the tube. The hexane was evaporated to dryness under nitrogen gas. The vitamin extract was then redissolved with 0.4 ml of chloroform and from this a 0.2 ml solution was taken in a microcell and placed in the spectrophotometer. Then 0.1 ml of TFA was rapidly added to the microcell and the solution was carefully but rapidly mixed by drawing up into the pipette and releasing back into the cell. The optical density was measured at 620 nm approximately 15 seconds after the addition of TFA. Detailed calculations for the vitamin A estimation are shown in appendix-I.

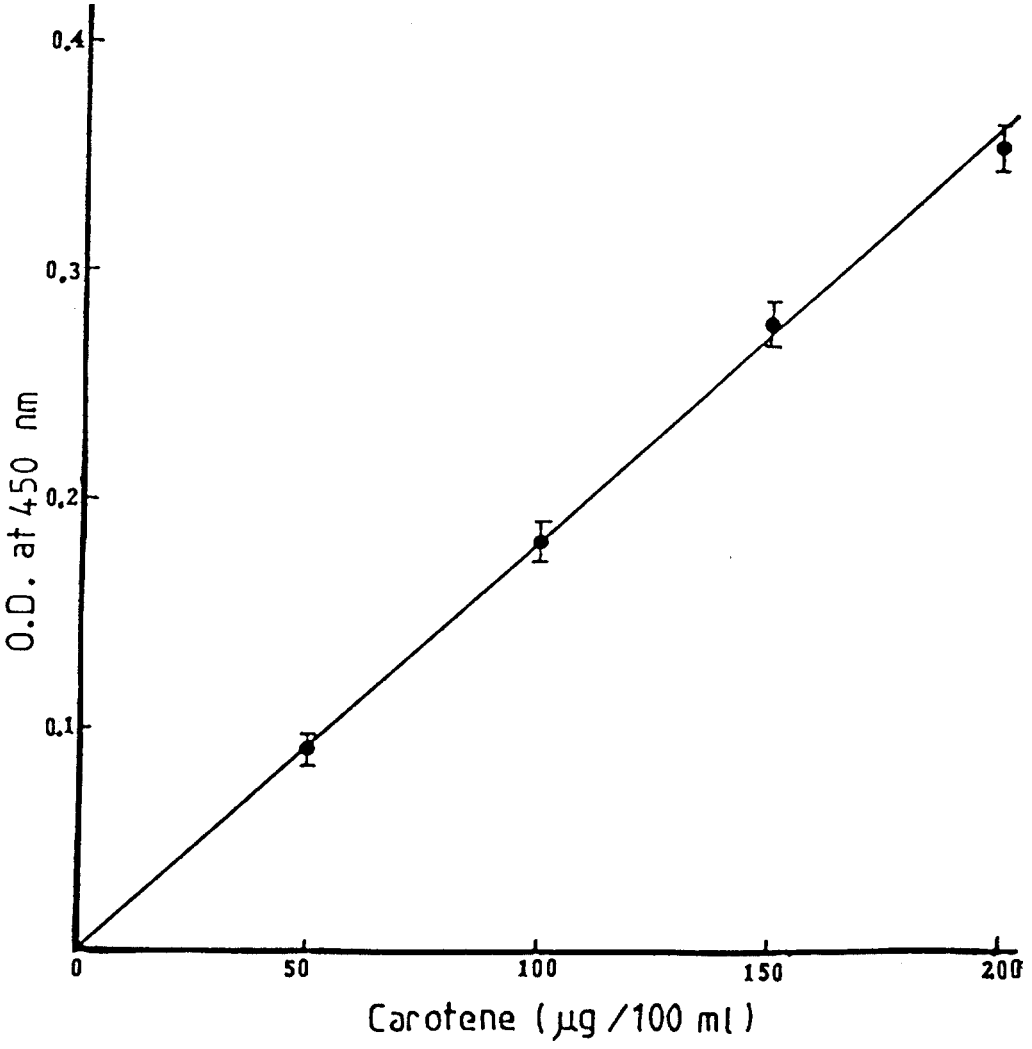


Figure: 2.2. Standard curve for serum β-carotene. Each point indicates the mean of three readings±S.E.

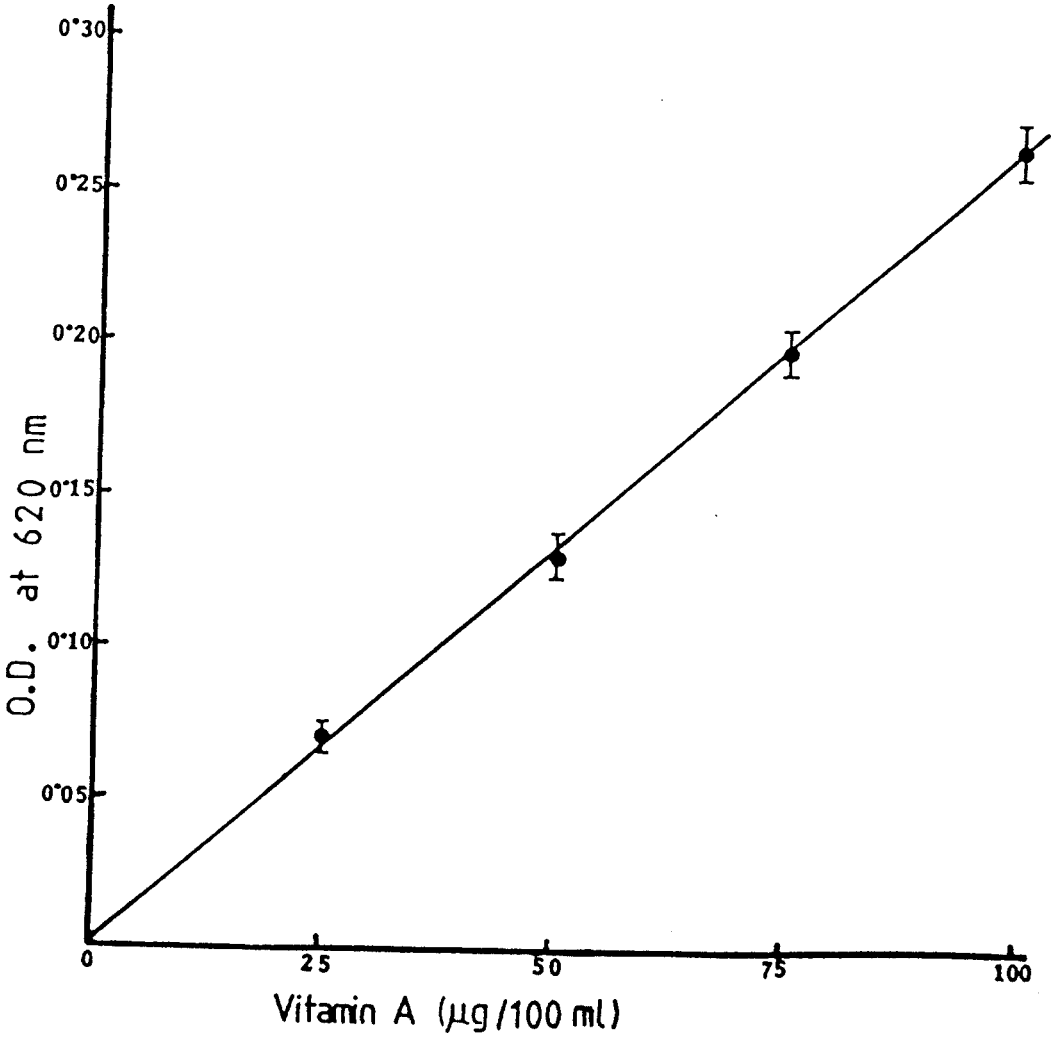


Figure: 2.3. Standard curve for serum vitamin A. Each point indicates the mean of three readings±S.E.

2.4.2. Estimation of serum vitamin A by high pressure liquid chromatography (HPLC):

Serum vitamin A was measured by HPLC by a modification of the method of Bieri et al.(1979).

2.4.2.1. Instrument for HPLC:

A solvent delivery system or pump (model 400 Spectroflow) and programmable absorbance detector (model 783 Spectroflow) were obtained from KRATOS Analytical Instruments. The autosampler was obtained from Spectraphysics (model SP 8780XR) and the Integrator from Spectraphysics (model SP 4290).

2.4.2.2. Materials:

The primary column was 25.0 cm X 4.5 mm stainless steel, packed with C^{18} silica (Spherisorb ODS2). The particle size was 5.0 µ. A guard column 2.0 cm X 0.4 cm packed with pellicular OD silica was attached to the primary column.

The solvent was HPLC reagent grade methanol (Rathburn Chemicals Ltd,Scotland): Water at 97.5: 2.5 ratio was used. There was 100.0 µl on a full loop injection mode.

Hexane HPLC grade (Rathburn Chemicals Ltd, Scotland.).

2.4.2.3. Standards :

Trans retinol (Aldrich Chemical Co, Ltd) was used as an external standard. Retinol acetate (Sigma) was used as an internal standard.

(a) Retinol standard (stock): 10.0 mg of retinol was dissolved in 100.0 ml methanol and was stored at -20°C.

(b) Working retinol standard: Stock retinol standard was diluted to the following concentrations ,2.5, 25.0, 50.0, and 75.0 µg/100.0 ml

solution. Working standard was freshly prepared before each experiment.

(c) Retinol acetate (Stock solution): 10.0 mg of retinol acetate dissolved in 100.0 ml methanol. The solution was stored at -20°C.

(d) Working retinol acetate standard: 0.6 ml of stock retinol acetate diluted to 100.0 ml methanol to give 60.0 µg/100 ml.

2.4.2.4. Procedure:

2.4.2.4.a. Retention time:

To determine the retention time, retinol and retinol acetate were injected into the HPLC instrument separately at a concentration of 100.0 µg/100.0 ml. The retention time was measured for each and is shown in the figure: 2.4. Both retinol and retinol acetate were then mixed at a same concentration and injected into the instrument. The peaks obtained from these two compound are also shown in figure: 2.4.

2.4.2.4.b. Standard curve:

To prepare the standard curve for peak height ratio, a constant amount of retinol acetate (60.0 µg/100.0 ml) was combined with a variable amount of working retinol solution and injected into the HPLC instrument and the peak height ratio measured by the integrator. The standard curve is shown in figure : 2.5 for external standard.

2.4.2.4.c. Analysis of serum retinol:

100.0 µl of serum sample was mixed with 100.0 µl of the internal standard solution (retinol acetate solution in methanol). The contents were mixed on a vortex mixer for 15 seconds. For extraction of lipid, 500.0 µl of hexane was added and mixed for 45 seconds in a vortex mixer. The tubes were centrifuged in a microfuge at 2000 X g

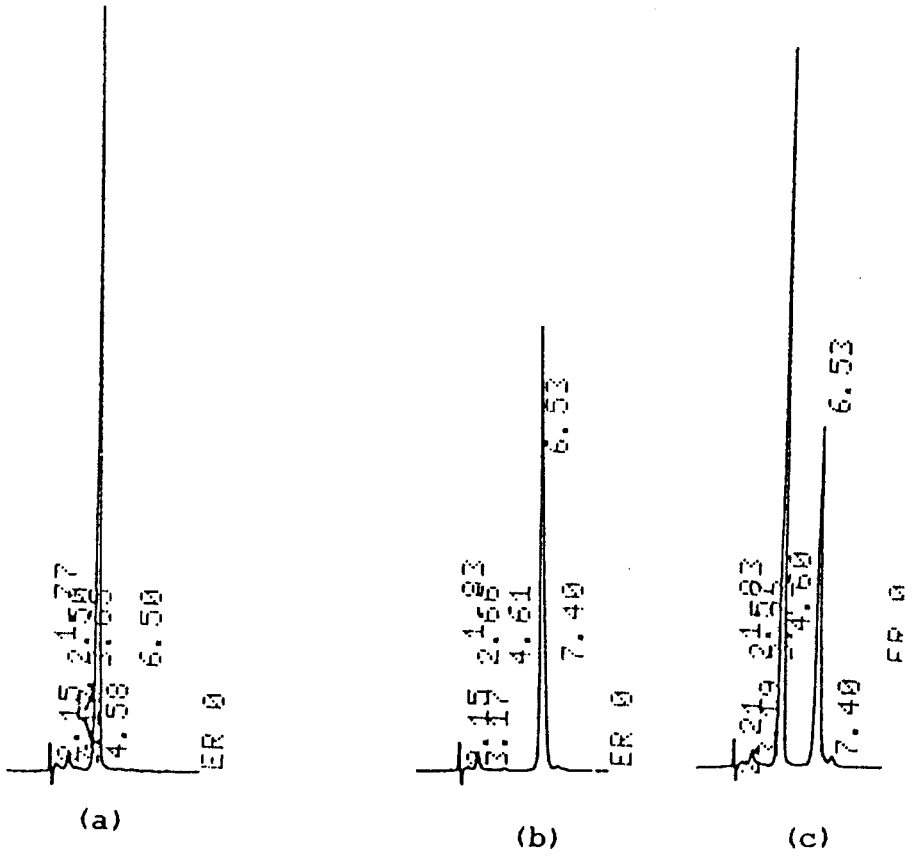


Figure: 2.4. Shows the peaks obtained with retinol (a) and retinyl acetate (b) when injected separately into the HPLC instrument. (c) Shows the peak obtained by retinol and retinyl acetate when injected together into the HPLC instrument. The retention time for retinol and retinol acetate are 4.58 and 6.53 respectively.

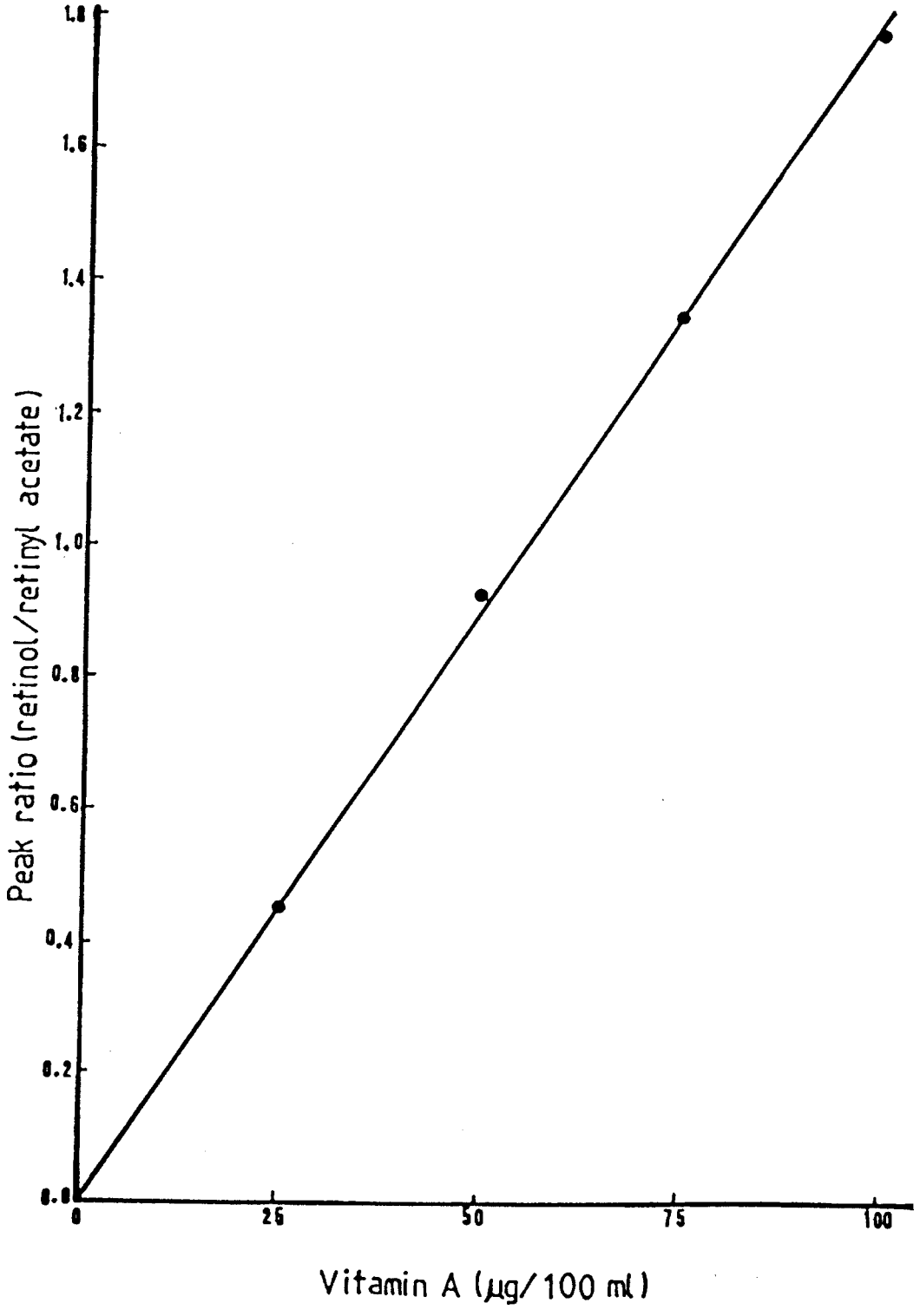


Figure: 2.5. Standard curve for vitamin A measured by HPLC. Each point indicates the mean of two readings.

for 2-3 minutes and the top hexane layer was collected in the HPLC vial. The hexane was evaporated under a stream of nitrogen in a 40°C water bath.

The vitamin A extract was then redisolved with 400.0 µl of methanol. The vial then put in the HPLC instrument and 100.0 µl of solution was injected. A flow rate of 1.0 ml/minute was used with the detector set at 0.01 attenuation. The O.D. was measured at 325 nm by the detector which was integrated by the programmed integrator. The amount of retinol was obtained by using the peak height ratio of the sample retinol to internal standard from the standard curve.

2.5. Histological methods:

The histology of gut and various lymphoid organs was examined, using the standard laboratory method for haematoxylin and eosin (H & E) staining.

2.5.1. Reagents:

(a) 10 % neutral buffered formalin (NBF): 4.0 g sodium dihydrogen orthophosphate and 6.5 g disodium hydrogen phosphate (anhydrous) were dissolved in distilled water and made up to 900.0 ml, then 100.0 ml formalin was added.

(b) Haematoxylin (BDH) solution: 5.0 g haematoxylin was dissolved in 50.0 ml of ethanol at 56°C, then 100.0 g potassium alum was dissolved in 950.0 ml of distilled water using low heat with frequent stirring. The alcoholic haematoxylin solution was then added to the hot alum solution, boiled and stirred. Just before turning off the heat, 2.5 g of mercuric oxide was added. Then the solution was cooled by plunging the container into cold water and 4.0 ml acetic acid was added before filtering. The solution was ready for immediate use.

(c) Eosin (Raymond Lamb): 1 % aqueous eosin.

(d) 1 % acid alcohol : 1.0 ml Conc. hydrochloric acid was added to 100ml of 70.0 % alcohol.

(e) Xylene.

(f) Absolute alcohol.

(g) 70.0 % alcohol.

2.5.2. Processing of paraffin sections:

Slices of spleen, thymus, mesenteric lymph node (MLN), gut and Peyer's patches from all groups of mice were fixed in 10% NBF for 24 to 48 hours and processed using a 16 hour schedule through alcohol and chloroform to paraffin wax on an automatic tissue processor. The tissues were embedded in Paramat paraffin wax (BDH). Sections were cut at 4 µ thickness with a Leitz rotary microtome, floated on to water at 50°C and mounted on clean glass slides without adhesive. The sections were then dried at 37°C in an incubator.

2.5.3. Staining procedure:

Slides were dewaxed using xylene, absolute alcohol, 70 % alcohol, washed in running tap water for 3 minutes and then stained in haematoxylin for 5 minutes. Excess stain was removed by washing in running tap water. The haematoxylin staining was differentiated by using 1.0 % acid alcohol for 5 seconds and then running tap water for at least 10 minutes. Thereafter the slides were stained in eosin for 5 minutes and rinsed briefly in tap water. Slides were dehydrated rapidly in a staining rack through 70 % alcohol, absolute alcohol (5 seconds in each dish) and then cleared in xylene for 5 seconds and mounted in D.P.X.

2.6. Mucin staining:

Equivalent portions of duodenum were examined for mucin content on the surface of epithelium and in the goblet cells by the method of Cook (1972).

All specimens were fixed in 10 % NBF, embedded in paraffin

wax and sectioned at 4 μ . Sections were dewaxed as described in the histological methods in section 2.5.2.

2.6.1. Reagents:

(a) Alcian blue (Raymond Lamb) solution: 1.0 g alcian blue dissolved in 3 % acetic acid solution to make the volume upto 100 ml.
(b) Neutral red (BDH): 0.5 % aqueous neutral red.
(c) Periodic acid (Sigma): 1.0 % aqueous periodic acid.
(d) Haematoxylin Solution (BDH).
(e) Schiff's reagent: 1.0 g Pararosaniline hydrochloride (sigma) was dissolved in 200 ml boiling distilled water and the solution allowed to cool to 50°C, 2.0 g potassium metabisulphite was then added. The solution was further cooled to room temperature and 2.0 ml HCl added. Finally 2.0 g activated charcoal was added and the solution kept overnight in the dark at room temperature. It was then filtered through No. 1 Whatman paper and stored in a dark container at 4°C.

2.6.2. Alcian blue method:

Dewaxed sections were stained with 1.0 % alcian blue in 3.0 % acetic acid for 5 minutes. Washed with distilled water and then counterstained with 0.5 % aqueous neutral red for 2-3 minutes, dehydrated, cleared and mounted.

2.6.3. Periodic acid Schiff's (PAS) method:

Dewaxed sections were treated with 1.0 % aqueous periodic acid for 2 minutes, rinsed with distilled water and then treated with Schiff's reagent for 8 minutes. Washed in running tap water for 10 minutes, then stained with haematoxylin for 10 seconds and finally washed in running tap water for 5 minutes. The sections were then dehydrated, cleared and mounted.

2.6.4. Combined alcian blue- (PAS) method:

Dewaxed sections were stained with alcian blue solution for 5 minutes, washed in distilled water and then treated with the periodic acid solution for 2 minutes. After washing in distilled water the sections were treated with Schiff's reagent for 8 minutes, washed in running tap water for 10 minutes and finally dehydrated, cleared and mounted.

2.7. Immunocytochemical studies:

B and T-lymphocytes of different lymphoid organs were stained by using the immunoperoxidase technique of Nakane and Pierce (1966).

2.7.1. Reagents:

(a) Liquid Nitrogen.
(b) Isopentane.
(c) Acetone.
(d) Rabbit anti mouse Ig peroxidase conjugate (DAKO, Denmark).
(e) Rat anti mouse Pan-T (Nordic Immunological Laboratories).
(f) Rabbit anti rat peroxidase conjugate (Nordic Immunological Laboratories).
(g) Tris buffered saline (TBS), pH-7.6: 80.0 g of sodium chloride, 6.05 g of Tris hydroxy methyl amine (TRIS) and 38.0 ml of 1(N) hydrochloric acid dissolved in 10 litre distilled water.
(h) Tris/ HCl buffer, pH -7.6 : 12.0 ml of 0.2 (M) Tris and 19.0 ml of 0.1 (N) HCl was added to 19.0 ml distilled water.
(i) DAB Substrate: 5.0 mg 3,3' diaminobenzidine tetrahydrochloride (Sigma) was dissolved in 10.0 ml Tris/ HCl buffer, pH-7.6. Immediately before use 100.0 µl of freshly prepared 1.0 % hydrogen peroxide was added.

2.7.2. Preparation of frozen sections:

A portion of spleen, and Peyer's patches from all groups of mice were wrapped in aluminium foil. These were snap frozen in liquid nitrogen and isopentane and stored in nitrogen until required for staining. When required for sectioning the tissues were mounted on to metal chucks using O.C.T. medium (Lamb, London). Frozen sections were cut on a cryostat, model T.E.(Slee Medical Equipment, London) and mounted on acid alcohol washed glass slides. After drying for at least 30 minutes at room temperature the slides were put in metal racks and placed in boxes containing silica gel which were sealed with an airtight lid and stored at -20°C. For immunostaining sections were allowed to dry at room temperature, fixed in water free acetone for 15 minutes, removed and washed twice in TBS after allowing the acetone to evaporate before proceeding with the immunohistochemical techniques.

2.7.3. B-Lymphocyte staining:

Rabbit anti mouse Ig peroxidase conjugate at its optimum dilution in TBS (pH-7.6) was applied to the section for 30 minutes. The slides were washed three times with TBS. A freshly prepared solution of substrate was then applied for 10 minutes. The slides were counterstained with haematoxylin for 2 minutes and differentiated with 1% acid alcohol, before finally dehydrating and mounting.

2.7.4. T- Lymphocyte staining:

Rat anti mouse Pan-T at its optimum dilution in TBS (pH-7.6) was applied to the sections for 30 minutes. The slides were washed three times with TBS. Then rabbit anti rat peroxidase-conjugate at an appropriate dilution with TBS was applied as a second antibody for 30 minutes. Thereafter the slides were washed with TBS three times. Substrate was added to the section for 10 minutes and washed again with TBS, counterstained with haematoxylin and finally differentiated

with 1.0 % acid alcohol for 8 seconds, dehydrated and mounted.

2.8. Measurement of total immunoglobulins specific to rotavirus antibodies in serum:

2.8.1. Cell-Enzyme linked immunosorbent assay (Cell-ELISA):

Total serum antibody specific to rotavirus was measured by using the modified method of McLean et al (1980).

2.8.1.1. Reagents:

(a) Complete tissue culture media (1X RPMI 1640, Flow laboratories, U.K.): 1X RPMI 1640 media 500.0 ml, Fetal calf serum 50.0 ml and 200 mM glutamine were mixed. Then 0.5 ml of penicillin (1,000,000 units/5.0 ml) and 0.5 ml streptomycin (1.0 g/5.0 ml) was added.
(b) 2.5 % Trypsin (GIBCO, U.K.).
(c) MA104 monkey kidney cells (Obtained from Birmingham Regional Virus Laboratory).
(d) SA11 simian rotavirus (Birmingham R.V.L.).
(e) Ascitic mouse rotavirus antibody (Birmingham R.V.L.).
(f) Rabbit anti mouse Ig peroxidase conjugate (DAKO, DENMARK).
(g) Phosphate buffered saline (PBS): 35.06 g of sodium chloride, 17.20 g of disodium hydrogen phosphate and 3.95 g of potassium dihydrogen phosphate were dissolved in distilled water to make 5 litre solution.
(h) Phosphate buffered saline-Bovine serum albumin (PBS-BSA) solution: 10.0 g of bovine serum albumin was dissolved in 1 litre PBS.
(i) Phosphate buffered saline-Tween solution: 0.5 ml tween 20 was mixed in 1 litre PBS.
(j) Phosphate-citrate buffer solution : 25.7 ml of 0.2 (M) diabasic sodium phosphate and 24.3 ml of 0.1 (M) citric acid were mixed with 50.0 ml of distilled water.
(k) OPD Substrate : Ortho-Phenylenediamine,20.0 mg and hydrogen peroxide (30% v/v),40 µl were mixed in 100.0 ml of phosphate-citrate

buffer.

2.8.1.2. Preparation of confluent monolayers of monkey kidney epithelial cells (MA104):

MA104 monkey kidney cells were grown in tissue culture flasks using RPMI 1640 medium with glutamine and 10% fetal calf serum (complete media). To harvest the cells, the medium was poured from the flask and the cells washed with serum free medium once. The cells were treated with 0.25% trypsin for 30-45 minutes at 37°C. The cell suspension were collected in sterile vials and centrifuged at 400 X g for 20 minutes at room temperature. The liquid was then discarded, and the cells adjusted to give a suspension of 10^4 cells/ml in complete medium. Finally 200 µl of the cell suspension was put into each well of a 96 well, flat bottomed tissue culture tray and incubated at 37°C for 48 hours.

2.8.1.3. Antibody assay:

After the growth of confluent monolayers of monkey kidney cells all 96 wells were infected with 1:50 dilution of SA11 simian rotavirus stock. The plates were further incubated at 37°C overnight. The plates were washed once with PBS-Tween and dried in air. The cells were then fixed with methanol for 5 minutes and air dried. The plates were incubated at 37°C for an hour with 100.0 µl/well of PBS-BSA solution. The plates were then washed once.

Thereafter, monoclonal rotavirus antibody (prepared 1:125 dilution) was used as a standard in the plate serially diluted with PBS. Serum samples were diluted appropriately and incubated for 90 minutes at room temperature and washed with PBS-Tween20 solution 5 times. Then 100 µl of rabbit anti mouse Ig peroxidase conjugate at appropriate dilution in PBS-BSA buffer was added to each well and incubated for 90 minutes. After 5 further washes with PBS-Tween followed by the addition of 100 µl of substrate /well and 30 minutes

incubation the reaction was stopped by adding 50.0 µl/well of 2.5 (M) sulphuric acid.

The optical density was measured in micro ELISA reader (model MR 580) at 490 nm. The end point of antibody titre was determined by using the O.D. value of monoclonal rotavirus antibody at a dilution of 1:16000 in all plates. The standard curve, prepared by using monoclonal rotavirus antibodies, is shown in figure: 2.6.

2.8.2. Enzyme linked immunosorbent assay (ELISA):

In the cell-ELISA method we observed that during the washing procedure cells were lost from the plates in a variable fashion with resulting variability in the final antibody measurement. In order to get around this problem a modified technique was developed for the measurement of serum rotavirus antibody by using direct coating of rotavirus antigen onto the plates.

2.8.2.1. Reagents:

(a) Protein coupling Buffer (pH-9.4): 1.59 g of sodium carbonate and 2.93 g of sodium hydrogen carbonate were dissolved in distilled water to make 1 litre solution.

All other reagent described in previous section:(2.8.1.1).

2.8.2.2. SA11 simian Rotavirus passage:

MA104 monkey kidney cells were grown in a 250 ml tissue culture flasks in complete media. After confluent growth was achieved, the medium was discarded and the flask was washed with PBS. SA11 simian rotavirus stock, 0.5 ml was added to 10.0 ml of fetal calf serum (FCS) free media poured into the flask , and incubated for 1 hour at 37°C. Thereafter the media was replaced by 2 % FCS medium and incubated for 4-8 hours at 37°C. This medium was discarded and the

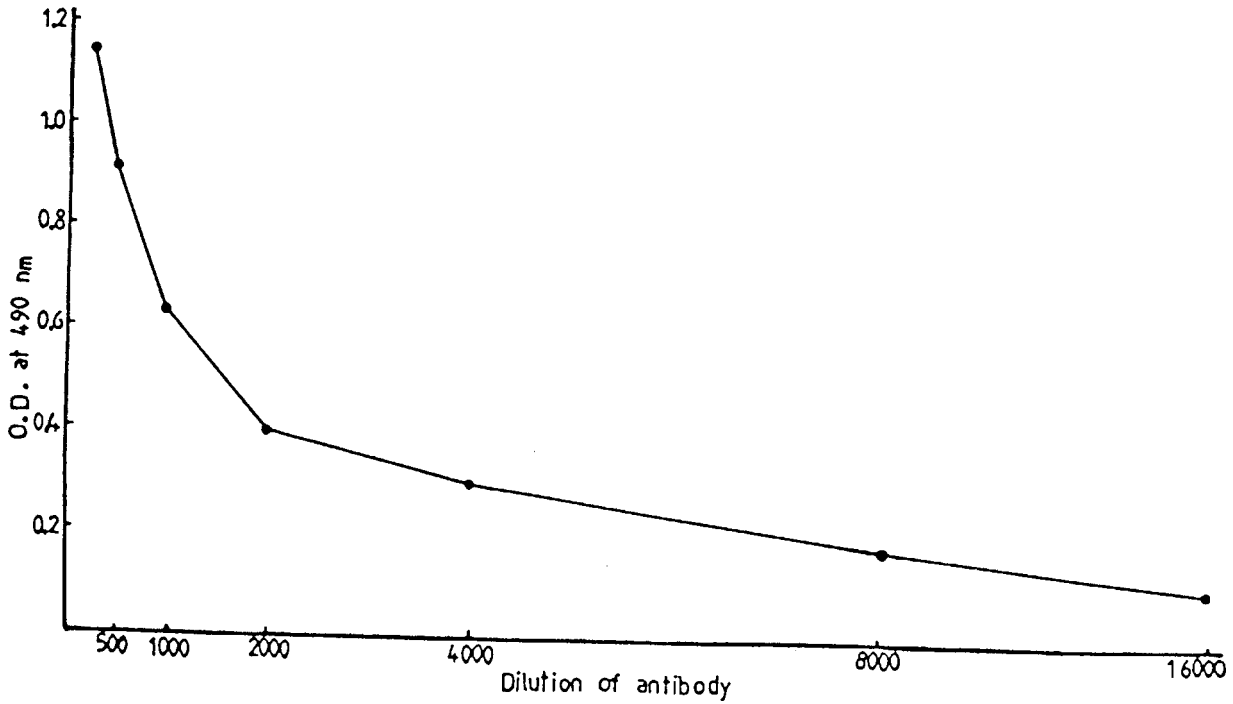


Figure: 2.6. Standard curve for the rotavirus antibody assay by cell-ELISA using monoclonal rotavirus antibody. Each point indicates the mean of two readings.

flask was washed with PBS. Finally, this was discarded and 10-20 ml serum free medium with 1 µg/ml trypsin was added and incubated at 37°C for 2-3 days depending upon the appearance of the full cytopathic effect. The cells were collected, then freeze/thawed three times. The cell debris was spun down at 400 X g for 10 minutes. The supernatent was collected and finally stored frozen in aliquots at -70°C.

2.8.2.3. Antibody assay:

100.0 µl of an optimum dilution of simian rotavirus (SA11) in protein coupling buffer was added to each well of a 96 well polyvinyl microtitre plate and incubated at 37°C for one hour. Then the plates were incubated at 4°C overnight. The plates were washed with PBS-Tween once and incubated at 37°C for one hour with 100.0 µl/well of PBS-BSA solutin. The plates were then washed once more.

Thereafter, monoclonal rotavirus antibody (prepared 1:125 dilution in PBS-BSA) was used as a standard and serially diluted in the plate with PBS. Serum sample were diluted appropriately and incubated for 90 minutes at room temperature and washed with PBS-Tween20 solution 5 times. Then 100 µl of rabbit anti mouse Ig peroxidase conjugate at appropriate dilution in PBS-BSA buffer was added to each well and incubated for 90 minutes. After 5 further washes with PBS-Tween 100 µl of substrate was added to each well and the wells were incubated for 30 minutes. The reaction was stopped by adding 50.0 µl/well of 2.5 (M) sulphuric acid.

Optical density was measured in a micro ELISA reader (model MR 580) at 490 nm. The units of anti-rotavirus activity are defined for the standard serum as dilution of serum X($1x10^6$). Hence the anti-rotavirus serum has 125 anti-rotavirus units at a dilution of 1/8000 and 62.5 units at dilution of 1/16000. For each serum under test a doubling dilution curve was assayed by ELISA. The antibody units present were then extrapolated from the standard curve using the lowest dilution of test serum for which the O.D. value lies on the linear portion of the standard curve. The standard curve was prepared

by using monoclonal rotavirus antibodies is shown in figure: 2.7. Detailed calculations for the antibody titres are shown in appendix-II.

2.9. Delayed-type hypersensitivity response:

The method for the measurement of delayed-type hypersensitivity was set up according to the method of Prof. Geoffrey L Asherson, Clinical Research Centre, Harrow, London , who is routinely using this method in his laboratory.

2.9.1. Reagent:

(a) Trinitro chlorobenzene (Picryl chloride).
(b) Acetone.
(c) Alcohol.
(d) Olive oil.
(e) Antigen concentration for immunization (5.0 % Picryl chloride): Picryl chloride, 5.0 g was dissolved in 10.0 ml acetone and then alcohol was added to make 100.0 ml solution.
(f) Antigen concentration for challenge (1.0 % Picryl chloride): Picryl chloride, 50.0 mg was dissolved in 0.5 ml acetone and then olive oil was added to make 5.0 ml solution.

2.9.2. Procedure:

On day 0, animals were immunized with 150.0 µl of 5.0 % Picryl chloride painted onto the shaved abdomen. 50.0 µl was also applied to each of the four foot pads. Controls received acetone-alcohol solution only. On day 5, ear thickness was measured and then the ears challenged with 1.0 % Picryl chloride solution (10.0 µl was applied to both sides of both ears). One day later the ear thickness was measured and delayed hypersensitivity was quantified as a percent increase in ear thickness.

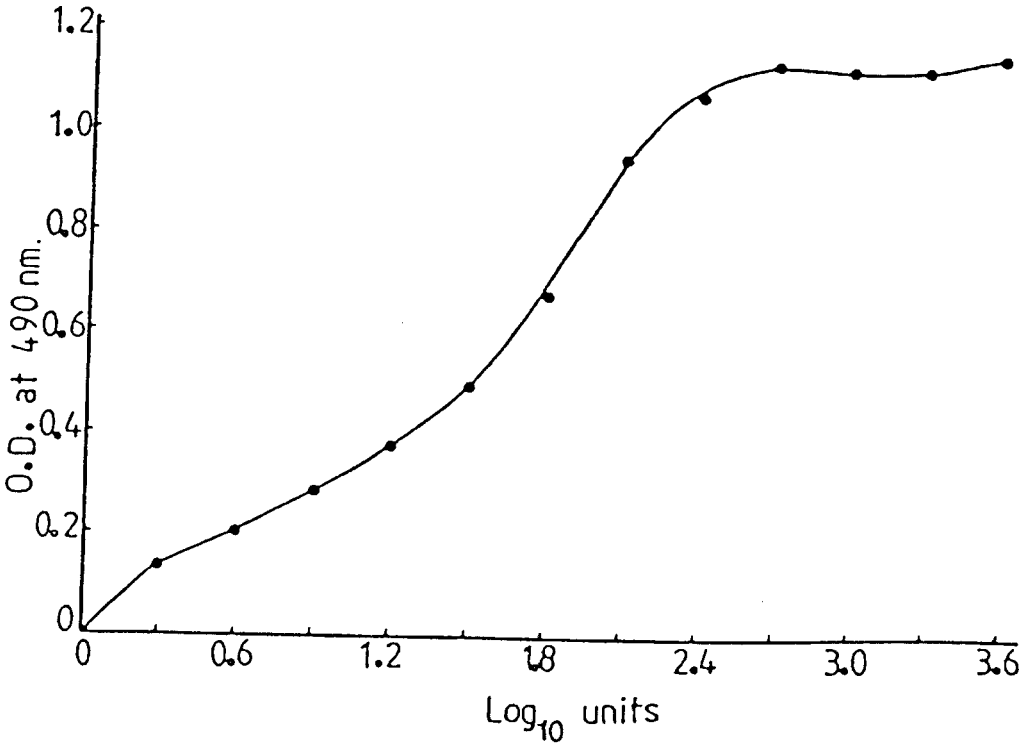


Figure: 2.7. Standard curve for the rotavirus antibody assay by ELISA using monoclonal rotavirus antibody. Each point indicates the mean of two readings.

2.10. Statistical analysis:

The result are expressed as a mean and standard error of the mean for each experimental group. Differences between groups were evaluated statistically using Student's "t" test, 2-way analysis of variance (ANOVA) for the parametric data and Wilcoxon rank sum test for nonparametric data (Goldstone, 1983).

The differences of the means were considered significant when $P<0.05$.

CHAPTER THREE

THE DEVELOPMENT OF A MOUSE MODEL FOR VITAMIN A DEFICIENCY

CHAPTER THREE

3.1. Introduction:

States of vitamin A nutriture have been defined as follows: deficient, marginal, satisfactory, excessive and toxic. Clinical manifestations may be present in the deficient and toxic states, but not in the other three. The satisfactory state, is characterized by the absence of any signs of vitamin A deficiency and by the presence of an adequate body reserve of the vitamin A. A liver concentration of 20.0 µg/g is considered to provide an adequate reserve (Olson,1987). When the body reserve of vitamin A is completely exhausted, the subjects are termed vitamin A deficient.

Indicators of vitamin A status can be divided into two major catagories: 1) Direct or primary, and 2) Indirect or secondary (Olson,1988). Direct indicators include the measurement of plasma (or serum) vitamin A, levels of vitamin A in tissues, the in vivo kinetics of administered deuterated vitamin A and the relative dose response. Indirect indicators include night blindness, skin disorders, a reduction of goblet cells in the intestine, conjunctiva and the other tissues . The cessation of body weight gain has also been considered as an indicator of vitamin A deficiency (Wolf,1980).

Vitamin A deficiency is thought to result primarily from an inadequate dietary intake of vitamin A. The usual method of producing vitamin A deficient animals has been simply to feed normal weanling animals a vitamin A free diet until growth ceases (Moore,1957). Normally these animals are fed the deficient diet for an additional 7-10 days in order to ensure the full development of deficiency. Most research workers have produced vitamin A deficiency in weanling rats (Nauss et al., 1979; Muto et al., 1972; Takagi and Nakano, 1983), in chicks (Bang and Foard, 1971) and in hamsters (DeLuca et al., 1975).

All these groups have defined established vitamin A deficiency by the vitamin A levels in liver or serum and by cessation of body weight gain. It is extremely difficult to produce a vitamin A deficient mouse model. McCarthy and Cerecedo (1952) have suggested that mice exhibit greater resistance with respect to body weight gain, life span and time of appearance of the symptoms of vitamin A deficiency. Smith et al (1987) have reported that feeding a vitamin A deficient diet to pregnant females during the 2nd week of gestation and continuing to feed their offspring the same diet produces vitamin A deficient mice at 8 weeks of age. They have suggested that rats and mice may differ with respect to vitamin A absorption, storage and metabolism.

Vitamin A deficiency induces loss of appetite, thus control animals tend to be well fed whereas the deficient animals are undernourished. Since vitamin A deficiency is accompanied by inanition, a result of low food intake during the later part of the depletion period (Takagi and Nakano,1983; Smith et al.,1987), secondary nutritional disorders may accompany a vitamin A deficient state.

The design of the present study sought to develop a suitable vitamin A deficient mouse model and differentiate the effect of vitamin A deficiency itself from the non-specific effects caused by a decreased food intake secondary to vitamin A deficiency. Therefore three groups of animals were used. a) control fed ad libitum, b) vitamin A deficient fed ad libitum and c) a pairfed group fed the control diet at the same intake as group b. The effect of vitamin A deficiency on growth and organs has been investigated. Finally, the liver and serum levels of vitamin A have been determined during the development of the deficient state.

3.2. Experimental procedure:

The protocol for the study is presented in figure-3.1 and the parameters studied are presented in figure-3.2. The routine procedures have already been described in chapter-2.

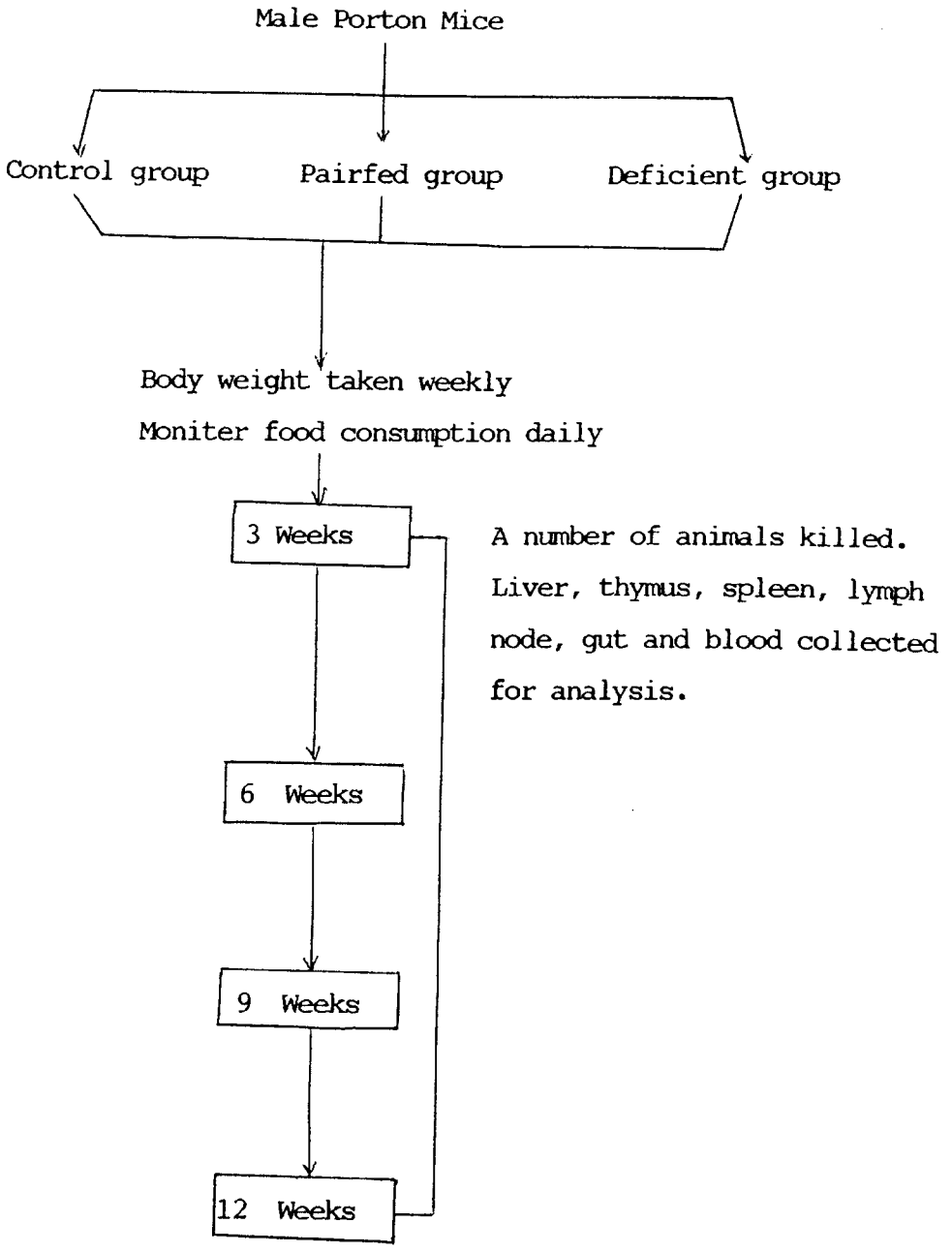


Figure:3.1 Scheme of the procedure for the study of the development of vitamin A deficiency in the mouse.

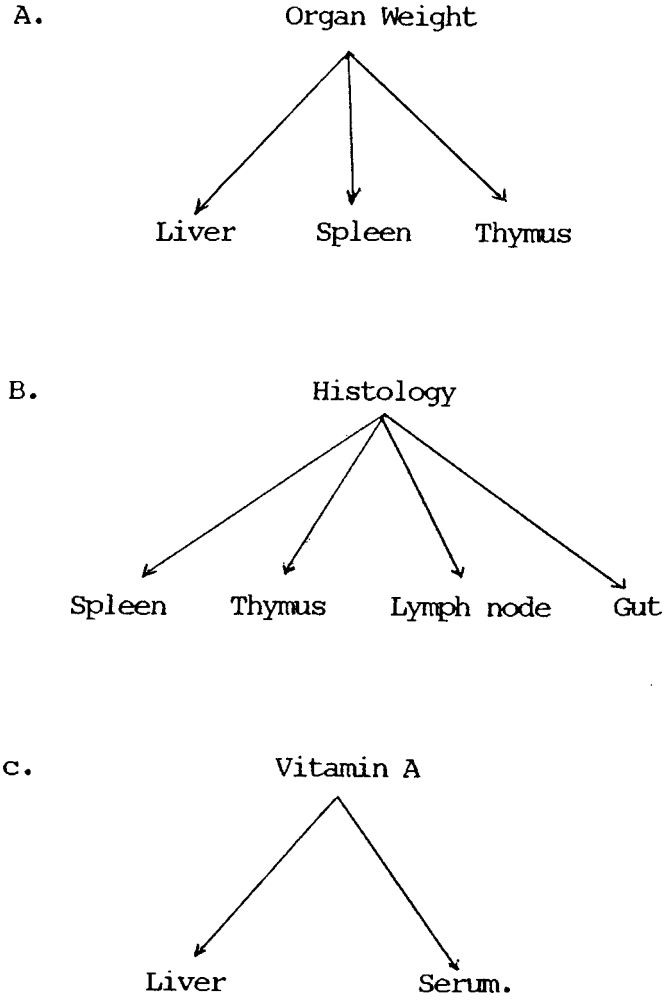


Figure:3.2 Outline of the specimens collected, and the investigations carried out during the development of vitamin A deficiency in the mouse.

3.2.1. Procedure for producing a vitamin A deficient model and the collection of samples:

Male Porton mice, 19-21 days old, were initially weight matched and divided into three groups, control, pairfed and vitamin A deficient. They were supplied with their respective diet as described in chapter-2.2. The animals were weighed weekly and food consumption was measured daily.

A number of animals were killed from each group after 3,6,9, and 12 weeks of feeding the experimental diet. The animals were bled by heart puncture and blood samples were allowed to stand at room temperature for one hour to allow clot formation. After clotting, the blood was centrifuged and the clear serum removed with a pasteur pipette. The serum samples were stored in ependorff tubes at -20°C until analysed.

Immediately after the blood was collected, the carcase of the animals was cut open and the liver, spleen and thymus were removed and weighed. The liver was then stored at -20°C until required for analysis. Spleen, thymus, a portion of small intestine with Peyer's patches and mesenteric lymph node were fixed in 10 % buffered formalin for histological analysis.

3.2.2. Determination of vitamin A:

3.2.2.1. Liver:

Liver vitamin A was determined at 3,6,9, and 12 weeks, after feeding the experimental diet by the method of Bayfield (1975) with the modifications described in chapter-2.3.

3.2.2.2. Serum:

As described in chapter- 2.4.1 serum vitamin A was estimated

at 3,6,9, and 12 weeks, by the method of Bradley and Hornbeck (1973) with slight modification.

3.2.3. Histological study:

The histology of the spleen, thymus, mesenteric lymph node and gut with Peyer's patches was examined after 3,6,9, and 12 weeks.

3.2.4. Statistical analysis:

Results are expressed as the mean and standard error of the mean. Differences between groups were evaluated statistically using Student's "t" test.

Difference in values were considered significant when $P < 0.05$.

3.3. Results and Discussions:

3.3.1. Body weights:

The body weights of the animals in all the groups are shown in figure-3.3 and 3.4. As animals were killed at different times the number of animals varies from 4 to 12 at the different time points in figure- 3.3. In figure-3.4 the mean body weights of the same four animals is shown for each point. This figure avoids any bias due to the killing of animals at different time intervals during the experiment.

The growth of the deficient mice began to diverge from that of the controls after 28 days on the deficient diet. The mice on the deficient diet alone ceased to grow after 9 weeks of feeding and their weight remained steady until the end of the experiment. The final body weights of the deficient animals were significantly lower than those of the control ($P < 0.01$) and the pairfed groups ($P < 0.05$). There was no significant difference in the body weight between the control and

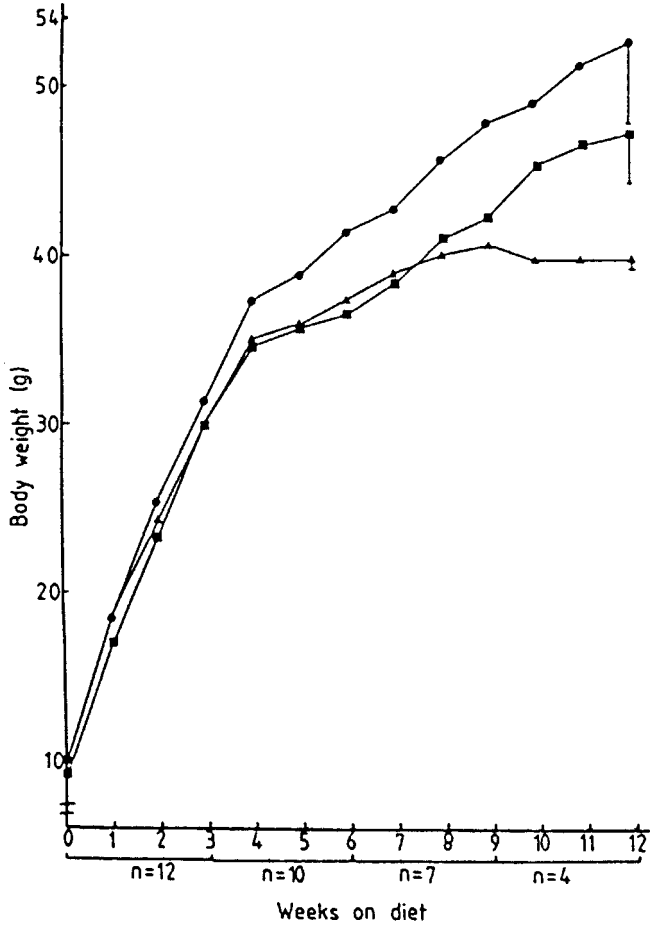


Figure:3.3 Body weight gain of different groups of animals during the expeiment. (•—•) control group, (■—■) pairfed group and (▲—▲) deficient group. Each point indicates the mean of a number of animals as given in the figure. At the 12 week time point, the mean± SEM is given.

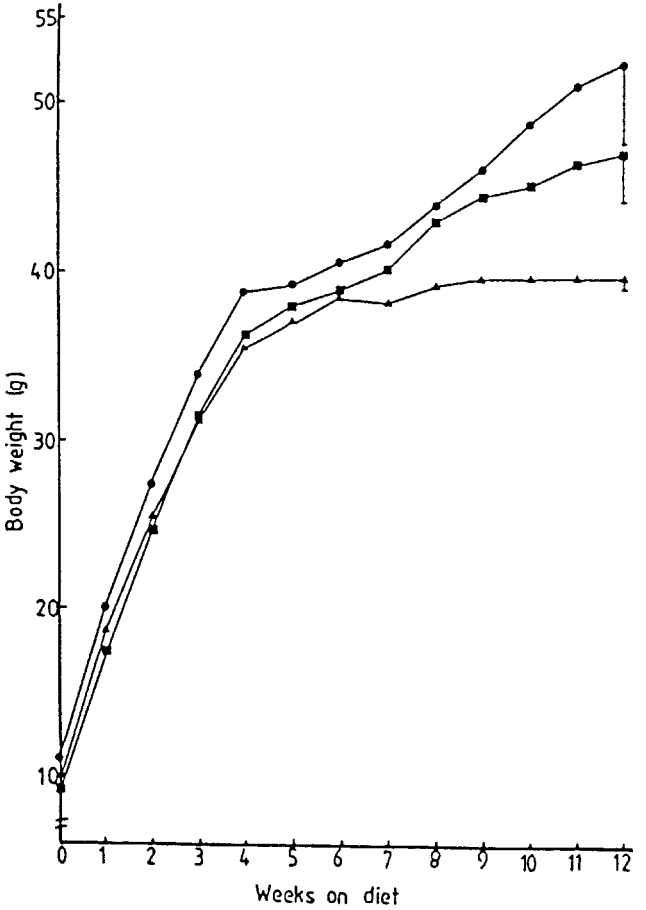


Figure:3.4 Body weight gain of different groups of animals during the experiment. Results shown are the mean of 4 animals in each group. (•—•) control group, (■—■) pairfed group and (▲—▲) deficient group. The 12 week time point represents the mean±SEM of 4 animals.

pairfed animals.

Our results are similar to those reported by Muto et al (1972), who used a similar diet. These authors showed that deficient animals ceased to grow after 50-60 days and exhibited a slight weight loss by the 75th day of the study. The animals in our study did not show any weight loss. Takagi and Nakano (1983) have shown that body weight gain in rats ceases approximately 40 days after feeding a vitamin A free diet. Nauss et al (1985) in their recent work have shown that rats fed a vitamin A deficient diet entered the weight plateau stage after 35 days of feeding. Smith et al (1987) reported that feeding a vitamin A deficient diet to pregnant females during the 2nd week of gestation and continuing to feed their offspring the same diet produces vitamin A deficient mice at 8 weeks of age. They have shown that at this age there was no difference in body weight between control and deficient animals. However, they have shown the loss of body weight in vitamin A deficient animals at about 11 weeks of age, when the animals also exhibited clinical signs of vitamin A deficiency. Since there is a direct relationship between growth rate and vitamin A utilization (Rechcigl et al.,1962), we consider that our animals became vitamin A deficient after 9-11 weeks of feeding. Although there is some disagreement concerning the time required for the development of the weight plateau stage between various studies, this discrepancy may be due to the liver reserve of the vitamin A or to the mode of utilization of vitamin A during post natal growth. Ganguly et al (1980) have described that weanling rats fed a vitamin A deficient diet continue to grow until their initial reserves of vitamin A are exhausted, at which time growth ceased and weight plateaued.

Another interesting result was demonstrated by Bieri (1969) with germ free rats. He observed that conventional animals reach a weight plateau in 32-35 days and that by 46 days all have died. In contrast, germ free vitamin A deficient animals on the same diet did not reach a weight plateau until 45-75 days and continued so for a long time without gaining or losing weight. None of these animals died

before 145 days. From the experiment of Bieri it is clear that vitamin A deficient animals reach a weight plateau but the time required for this depends on environmental conditions.

The ingredients of the diet used for the vitamin A deficient model may be another important factor which can play a role in the cessation of growth. Nauss et al (1985) have used dextrose and dextrin instead of starch and their animals reached the weight plateau after 35 days. Bieri (1969) used a more rigorously deficient diet in which casein was replaced by 18 L-amino acids and corn starch by sucrose. This diet led to an early weight plateau even in germ free condition, though one week later than the conventional rats.

When we used ordinary casein instead of vitamin free casein in one of our initial experiments, we observed that the vitamin A deficient group did not reach a weight plateau even at 12 weeks although they had a decreased rate of growth compared with controls. The levels of vitamin A in the liver of the deficient animals after 12 weeks were 6 µg/liver. At this point we discontinued the experiment and changed the diet composition.

The vitamin A deficient animals had a significantly lower body weight compared with the pairfed animals, although both groups had eaten the same amount of diet. This may be because vitamin A deficiency leads to a change in metabolic efficiency. Since growth results from a combination of an increase in cell number and cell mass, vitamin A deficiency may play a role in growth at a cellular level.

3.3.2. Diet intake:

The daily intakes of control and deficient mice are shown in figure : 3.5. The intake of the deficient animals became slightly lower than control animals after six weeks on the experimental diet. Nauss et al (1985) have reported that deficient rats had a lower intake of diet after 35-41 days. Krishnan et al (1976) also reported low food intake of deficient animals compared with controls. In

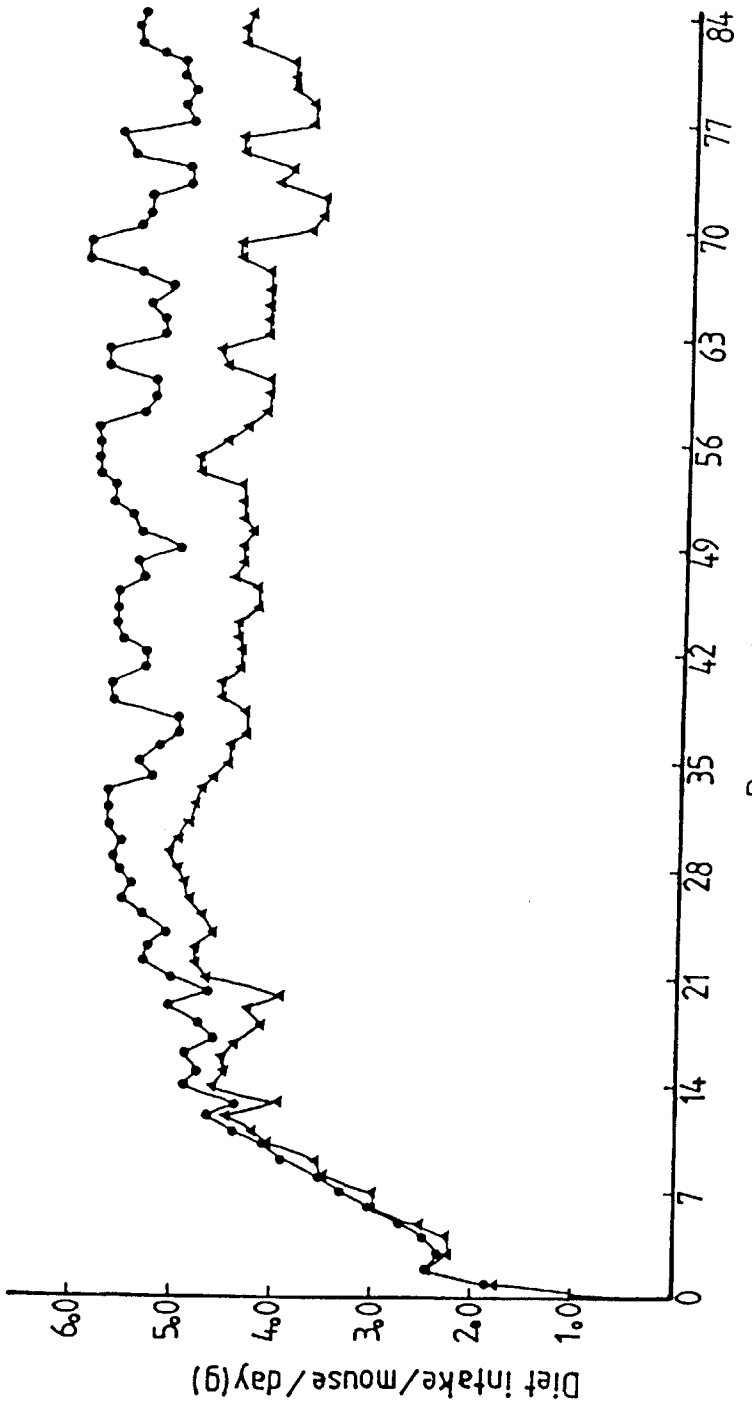


Figure 3.5 Average daily diet intake by control (●—●) and vitamin A deficient animals (▲—▲) during the experiment.

contrast, Takagi and Nakano (1983) have reported that although no anorexia was evident in their experimental animals there was a reduction in body weight gain at about 40 days. Smith et al (1987) have also shown that up to 8 weeks of age, vitamin A deficient mice had eaten the same amount as controls, thereafter deficient mice ate less.

Since vitamin A deficient mice have a tendency to eat less food than controls, we used a pairfed control group. The pairfed group was used to compensate for the potential effect of other nutritional deficiencies. The data from deficient animals are compared with the pairfed group rather than ad libitum controls. We are therefore in a position to differentiate the specific role of vitamin A deficiency rather than the combined effect of low food intake and vitamin A deficiency.

3.3.3. Organ weights:

3.3.3.1. Liver weight:

The liver weights at different time intervals during the experiment are shown in table: 3.1. The total liver weights of deficient animals after 9 weeks and 12 weeks on the experimental diet were significantly lower than those of controls ($P<0.05$). We observed no significant difference between pairfed and deficient groups. The liver weights of the pairfed group were significantly lower than the controls after 9 weeks of feeding ($P<0.001$) but not at 12 weeks. Our results are similar to the findings of Takagi and Nakano (1983) who have shown that the liver weights of vitamin A deficient animals were significantly lower than controls. They did not include pairfed controls in their experiment.

Nauss et al (1979) have shown that both the total and relative liver weights of control rats were significantly higher than pairfed control and deficient animals. Zile et al (1979) have also shown that both the total and relative liver weights of vitamin A

Table : 3.1

Effect of vitamin A deficiency on the liver weight (Mean±SEM)

a. Total weight.

Group	3 Week[1] (2) g	6 Week (3) g	9 Week (3) g	12 Week (4) g
Control	1.44	2.03±0.15	2.26±0.02	2.29±0.2
Pairfed	1.50	1.57±0.09	1.60±0.06**	1.78±0.09
Deficient	1.48	1.67±0.15	1.89±0.11*	1.71±0.07*

b. As % of body weight.

Group	3 Week[1] (2) %	6 Week (3) %	9 Week (3) %	12 Week (4) %
Control	5.47	5.01±0.22	4.55±0.13	4.35±0.07
Pairfed	5.38	4.59±0.25	4.14±0.07	3.80±0.07+
Deficient	5.68	4.89±0.20	4.58±0.37	4.30±0.20

[1] Only mean of two animals in each group.

Figures in the parantheses indicate sample size.

Significantly different from the corresponding control group by "t" test, *P<0.05; **P<0.001; +P<0.01.

deficient animals were significantly lower than controls. These authors controlled the food intake of the control group after 30 days of the dietary regimen so that they grew at the same rate as the deficient animals. They did not see any change in the liver composition (RNA or DNA content/g tissue) induced by lack of vitamin A during the period of growth. Our results, in contrast, show that there was no significant difference between the deficient and the two control groups in the relative liver weight as shown in table: 3.1.b. Although at 12 weeks pairfed animals had significantly lower relative liver weights compared with controls, this discrepancy reflects the small number of animals in each group.

The results presented indicate that the liver itself is not affected by vitamin A deficiency alone but that vitamin A deficiency along with low food intake affects liver by decreasing liver size. Low food intake alone does not have any effect on liver weight, the values for 9 weeks probably reflect the small number of animals in the study.

3.3.3.2. Spleen weight:

The spleen weights of all groups at different time intervals during the study are shown in table: 3.2. Whole spleen weights of deficient animals at 6 weeks were significantly lower than those of pairfed animals ($P<0.05$) but not of the ad libitum control group. After 12 weeks the spleen weights of the deficient animals were significantly higher than the pairfed group ($P<0.05$) but compared with the control group there was no significant change, although there is a tendency towards a higher spleen weight in the deficient animals. There is, therefore, no correlation between spleen weight and the progress of vitamin A deficiency.

The relative spleen weight (spleen weight as percentage of body weight) of vitamin A deficient animals after 12 weeks of feeding was significantly higher than control ($P<0.01$) and pairfed groups ($P<0.05$, see table: 3.2.b). Nauss et al (1979) have shown significantly higher total spleen weight in control rats compared

Table :3.2

Effect of vitamin A deficiecy on the spleen weight (Mean±SEM)

a. Total weight.

Group	3 Week[1] (2) mg	6 Week (3) mg	9 Week (3) mg	12 Week (4) mg
Control	107.5	98.7±9.9	104.2±3.7	104.5±4.5
Pairfed	115.0	99.0±2.1	100.0±2.7	97.0±0.4
Deficient	117.0	91.0±1.0"	98.0±7.0	143.0±16.5"

b. As % of body weight.

Group	3 Week (2) %	6 Week (3) %	9 Week (3) %	12 Week (4) %
Control	0.41	0.24±0.01	0.21±0.02	0.20±0.0""
Pairfed	0.41	0.29±0.02	0.26±0.02	0.21±0.01
Deficient	0.45	0.27±0.02	0.24±0.02	0.36±0.04"

[1] Mean of two samples in each group.

Figure in the parentheses indicate sample size.

"Significantly different from the corresponding pairfed group ($P<0.05$) by "t" test.

""Significantly different from the corresponding deficient group ($P<0.01$) by "t" test.

with pairfed and vitamin A deficient animals. These authors however found no significant change in relative spleen weights. Nauss et al (1985) have shown no significant changes in relative spleen weight between vitamin A deficient and pairfed control animals during the early or late stage of vitamin A deficiency. Takagi and Nakano (1983) found no change in spleen weight between vitamin A deficient and control groups. Zile et al (1979) have shown that both the total and relative spleen weights of vitamin A deficient animals were significantly lower than those of control animals. They have also shown that the cellularity of the vitamin A deficient spleen was lower by about one billion cells per gram tissue. In contrast, Smith et al (1987), have shown that there was no change in either spleen weight or total nucleated cells/organ in deficient animals as compared to controls at 8 weeks of age. However, they observed higher spleen weight at 11 weeks of age when the vitamin A deficient animals had eaten less diet than controls. This group have suggested that the raised spleen weight was due to the combined effect of vitamin A deficiency and inanition. Further they have provided information that this increase in spleen weight was due to an increase in B-lymphocytes in the spleen.

3.3.3.3. Thymus weight:

The thymus weights of all groups at different times during the feeding experiment are shown in table:3.3. The total thymus weights of vitamin A deficient animals taken at 6 weeks or later were found to be significantly lower than those of control and pairfed animals ($P<0.05$). The total thymus weights of the pairfed animals at 6 and 9 weeks were significantly lower than those of controls ($P<0.05$) but at 12 weeks no significant changes were found although pairfed animals had an apparently lower value. The thymus weights were also expressed as a percentage of body weight (table: 3.3b). The relative thymus weight of vitamin A deficient animals at 6 weeks and 9 weeks were significantly lower than those of control and pairfed animals

Table : 3.3

Effect of vitamin A deficiency on the thymus weight (Mean±SEM)

a. Total weight.

Group	3 Week[1] (2) mg	6 Week (3) mg	9 Week (3) mg	12 Week (4) mg
Control	100.5	67.7±1.8	56.3±3.2	54.8±5.3
Pairfed	83.5	49.3±1.2**	43.8±0.9**	46.8±7.6
Deficient	98.5	32.7±2.7*	31.0±2.1*	36.8±1.0*

b. As % of body weight.

Group	3 Week[1] (2) %	6 Week (3) %	9 Week (3) %	12 Week (4) %
Control	0.38	0.17±0.01	0.11±0.01	0.104±0.003
Pairfed	0.30	0.14±0.01	0.11±0.01	0.100±0.008
Deficient	0.38	0.10±0.01*	0.08±0.01*	0.093±0.003**

[1] Mean of two samples.

Figures in the parentheses indicate sample size.

* Significantly different from the corresponding control and pairfed group ($P<0.05$) by "t" test.

** Significantly different from the corresponding control group ($P<0.05$) by "t" test.

($P<0.05$). The 12 week deficient animals, however, had significantly lower relative thymus weight when compared with the control group ($P<0.05$) but not with the pairfed group. Nauss et al (1979) have shown that the total thymus weights of deficient animals were significantly lower than those of control and pairfed animals, which is in agreement with the data presented here. These authors did not, however, find any changes in relative thymus weight between vitamin A deficient and control groups. In contrast Krishnan et al (1974) have shown the relative thymus weights of vitamin A deficient animals to be significantly lower than the control and pairfed groups.

Our results indicate that thymus weight is affected by vitamin A deficiency both alone and in combination with low food intake, as the relative thymus weight of vitamin A deficient mice were significantly lower than those of control and pairfed animals at all time points other than 12 weeks. These results suggest that vitamin A deficiency, either alone, or associated with low food intake can lead to thymic atrophy. Low food intake alone did not lead to a significant reduction in the relative weight of the thymus. Zile et al (1979) have shown that the thymus gland is severely affected by the absence of vitamin A in the diet. They have shown that both the thymus weight and relative thymus weight of vitamin A deficient animals is reduced significantly when compared with controls. Further they have provided evidence that the decreased thymus weight is due to a decrease in cell number.

3.3.4. Vitamin A levels:

3.3.4.1. Liver vitamin A:

The percentage recovery achieved using our method for vitamin A estimation in mouse liver was estimated by adding extra vitamin A acetate to the liver homogenate. Estimations were carried out in duplicate. The results for percentage recovery are shown in table: 3.4. An overall recovery figure for vitamin A of 86.8 % was obtained

using this method.

Liver vitamin A, determined at different time intervals during the development of vitamin A deficiency, is shown in table: 3.5. Mice fed the control diet had normal vitamin A stores which increased steadily during the study. In contrast, mice fed the vitamin A deficient diet had low levels of vitamin A by week 3 and there was a gradual fall in the liver vitamin A level thereafter. At six weeks the liver vitamin A of the deficient animals was less than 2.0 µg/liver. Vitamin A levels of deficient animals were significantly lower than those of control and pairfed groups after 6 weeks on the experimental diet. The pairfed group also had significantly reduced levels of liver vitamin A after 6 and 9 weeks of feeding. After 12 weeks the vitamin A level of pairfed livers was low compared with controls but the difference was not significant. Smith and Hayes (1987) have shown that the liver retinyl palmitate concentration of vitamin A deficient mice at 8 weeks of age was <17.0 % of that of controls. This group started feeding the vitamin A deficient diet to pregnant females during the second week of gestation and continued to feed their offspring the same diet. The variations observed in their study could be explained by the large vitamin A stores of pregnant females which were thus able to supply sufficient vitamin A to the fetus and pups. Muto et al (1972) have suggested that adult animals take about 4-5 months to become vitamin A deficient because their liver vitamin A stores are high. Moreover, Smith and Haye's (1987) vitamin A deficient diet contained amino acid supplements (arginine, methionine, glycine and cystine) in addition to 18 % casein and they used a gel form of diet. Instead we used vitamin free casein but without amino acid supplements, as a pelleted diet. Nauss et al (1985) have shown that without supplementation with amino acids a diet containing vitamin A free casein could produce vitamin A deficient rats by 35-40 days of feeding.

Our animals were received at 19-21 days old and their initial liver vitamin A reserves were estimated at 10-15 µg/liver. The vitamin A deficient group therefore took 9 weeks to completely exhaust stored vitamin A.

Table : 3.4

Recovery of liver vitamin A

Sample analysed	Vitamin A[1] µg/g	% Recovery
Liver (0.5 g)	77.7	
Liver + Vitamin A acetate (0.5 g) (50.0 µg)	164.5	86.8

[1] Mean of two reading.

Table : 3.5

Effect of vitamin A deficiency on the liver vitamin A (Mean±SEM)

Group	3 Week[1] (2) µg/liver	6 Week (3) µg/Liver	9 Week (3) µg/Liver	12 Week (4) µg/Liver
Control	51.4	155.9±6.8	237.9±12.6	309.6±19.9
Pairfed	51.8	122.1±5.9""	195.5±1.8""	261.1±12.7
Deficient	5.7	1.6±1.1"	0.4±0.1"	0.5±0.1"

[1]Mean of two samples.

Figures in the parentheses indicate sample size.

"Significantly different from control and pairfed group by "t" test ($P < 0.001$).

""Significantly different from control group by "t" test ($P < 0.05$).

3.3.4.2. Serum vitamin A:

Percentage recovery estimates for vitamin A from serum were performed using three different samples and the results are shown in table: 3.6. Overall recovery of vitamin A was 87.6 %.

3.3.4.2.a. Serum carotene:

The serum carotene levels of all groups are shown in table: 3.7. Serum carotene levels were virtually undetectable in all groups. The results presented in this study are for β-carotene only, which is the major carotene in the blood. This finding is not unexpected as the diet used was almost vitamin A and carotene free, although the corn oil may have contained trace amounts of carotene.

3.3.4.2.b. Serum retinol:

Serum vitamin A levels of all the groups are shown in table:3.8. The serum vitamin A levels of the deficient group after 3 weeks on the diet were approximately 40% lower than those of control or pairfed animals. At six weeks the serum vitamin A levels of the deficient mice were 15 % of control values and thereafter remained at this level or slightly lower. After 6 weeks of feeding, serum vitamin A levels of the pairfed animals also appeared significantly lower than those of controls ($P<0.05$). These results may reflect the limited sensitivity of the spectrophotometric method which cannot distinguish low levels of vitamin A in the presence of background turbidity associated with the sample extraction method. Other investigators have reported less than 2 µg/100ml serum vitamin A after 30-40 days on a vitamin A free diet (Muto et al.,1972; Nauss et al.,1985). Therefore we have developed an HPLC method for the estimation of serum vitamin A. Smith and Hayes (1987) have shown that vitamin A deficient mice at the age of 8 weeks have about 20.0 % of serum vitamin A compared with controls.

Table : 3.6

Recovery of serum vitamin A

Sample	Vitamin A µg/100ml	Recovery %	Mean Recovery %
1	7.0		
2	52.4		
3	62.0		
1 + VA*	89.3	83.46	
2 + VA	136.0	89.24	87.6
3 + VA	146.0	90.12	

*VA = Vitamin A acetate (100 µl/100ml).

Table : 3.7

Effect of vitamin A deficiency on the serum carotene (Mean±SEM)

Group	3 Week[1] (2) µg/dl	6 Week (3) µg/dl	9 Week (3) µg/dl	12 Week (4) µg/dl
Control	3.0	2.6±0.2	2.6±0.2	2.6±0.2
Pairfed	2.7	2.2±0.2	2.6±0.2	2.2±0.2
Deficient	2.7	2.2±0.2	2.0±0.2	2.2±0.4

Table : 3.8

Effect of vitamin A deficiency on the serum vitamin A (Mean±SEM)

Group	3 Week[1] (2) µg/dl	6 Week (3) µg/dl	9 Week (3) µg/dl	12 Week (4) µg/dl
Control	31.45	39.6±0.7	39.9±1.0	45.8±1.5
Pairfed	28.90	32.2±1.3*	33.1±0.2*	37.2±0.8*
Deficient	17.30	6.0±0.9**	4.8±0.5**	5.3±1.0**

[1]Mean of two samples.

Figures in the parentheses indicate sample size.

*Significantly different (P <0.05) from control group by "t" test.

**Significantly different (P <0.005) from control and pairfed group by "t" test.

3.3.5. Histological study:

3.3.5.1. Spleen:

The histology of spleen at different time intervals during the progress of vitamin A deficiency was examined in all groups by light microscopy. Representative photographs of pairfed and vitamin A deficient animals after 12 weeks are shown in figure : 3.6. Photographs of the control animals are not shown as their histological appearance was similar to pairfed animals. Both the splenic red and white pulp of all groups appeared normal. In the white pulp, the periarteriolar lymphoid sheaths (PALS) are seen in various planes of section. PALS are readily identifiable by the presence of central arterioles. Around the central arterioles a pale T cell area was observed in all the three groups but without any significant change in size or area. The B cell areas contain many follicles, some with germinal centres. There was no significant difference in follicle size or structure between the three groups of mice. There was no apparent loss of lymphoid cells in vitamin A deficient animals. Krishnan et al (1974) have shown that the spleen of vitamin A deficient animals with low food intake showed marked involution of germinal centres and a variable degree of lymphocyte depletion. These changes were also found in pairfed control animals but were less marked.

3.3.5.2. Thymus:

The histology of the thymus of all groups was examined at different time intervals. Representative photographs of pairfed and vitamin A deficient animals are shown in figure: 3.7. The outer cortical area was found to be normal in all groups of animals and no involution was observed. The cortex of all groups show relatively large lymphocytes which appeared darkly stained. The medulla of all groups was also found to be normal in appearance. The areas of the

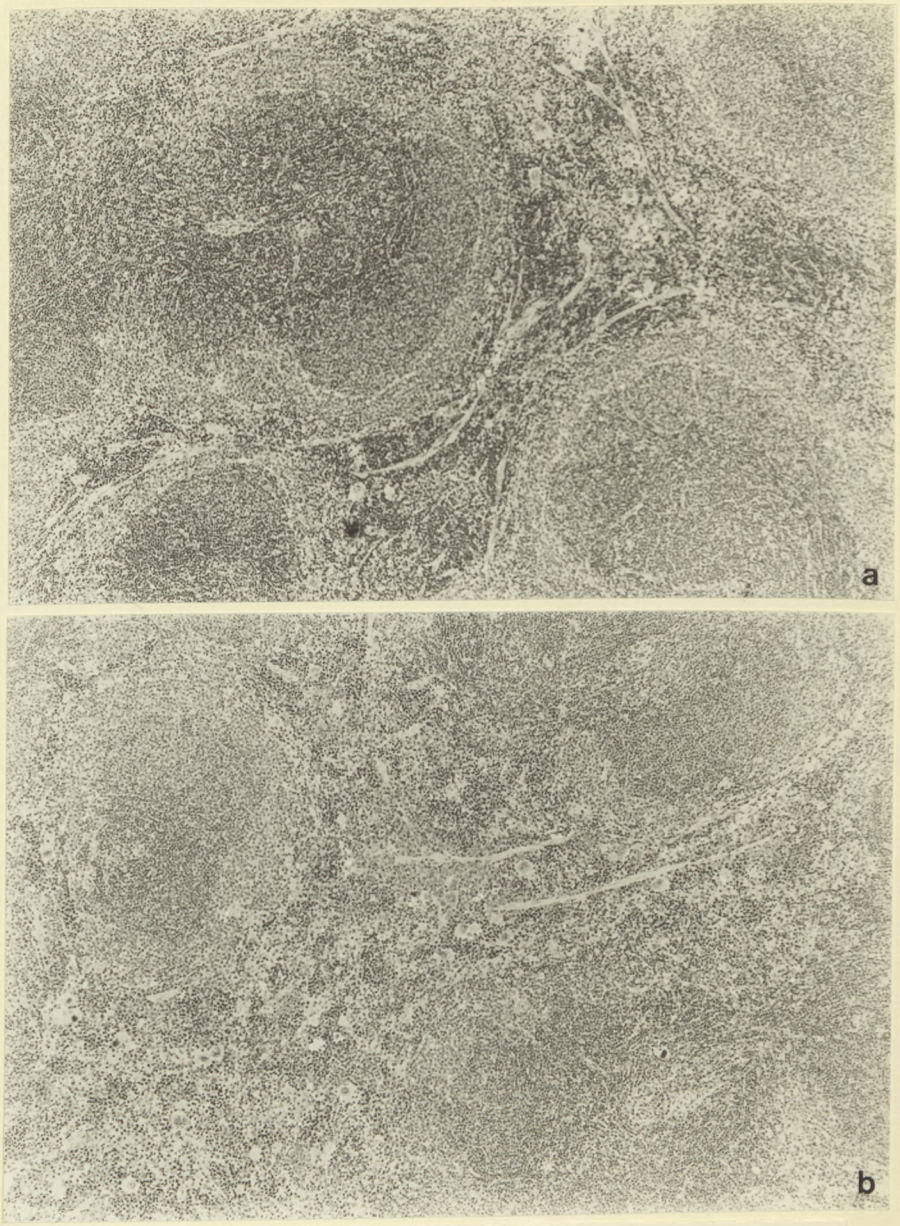


Figure: 3.6. Representative photographs of the histology of the spleen of (a) Pairfed and (b) vitamin A deficient mice. H & E stain, X 70. There is no difference in structure apparent between vitamin A and pairfed groups.

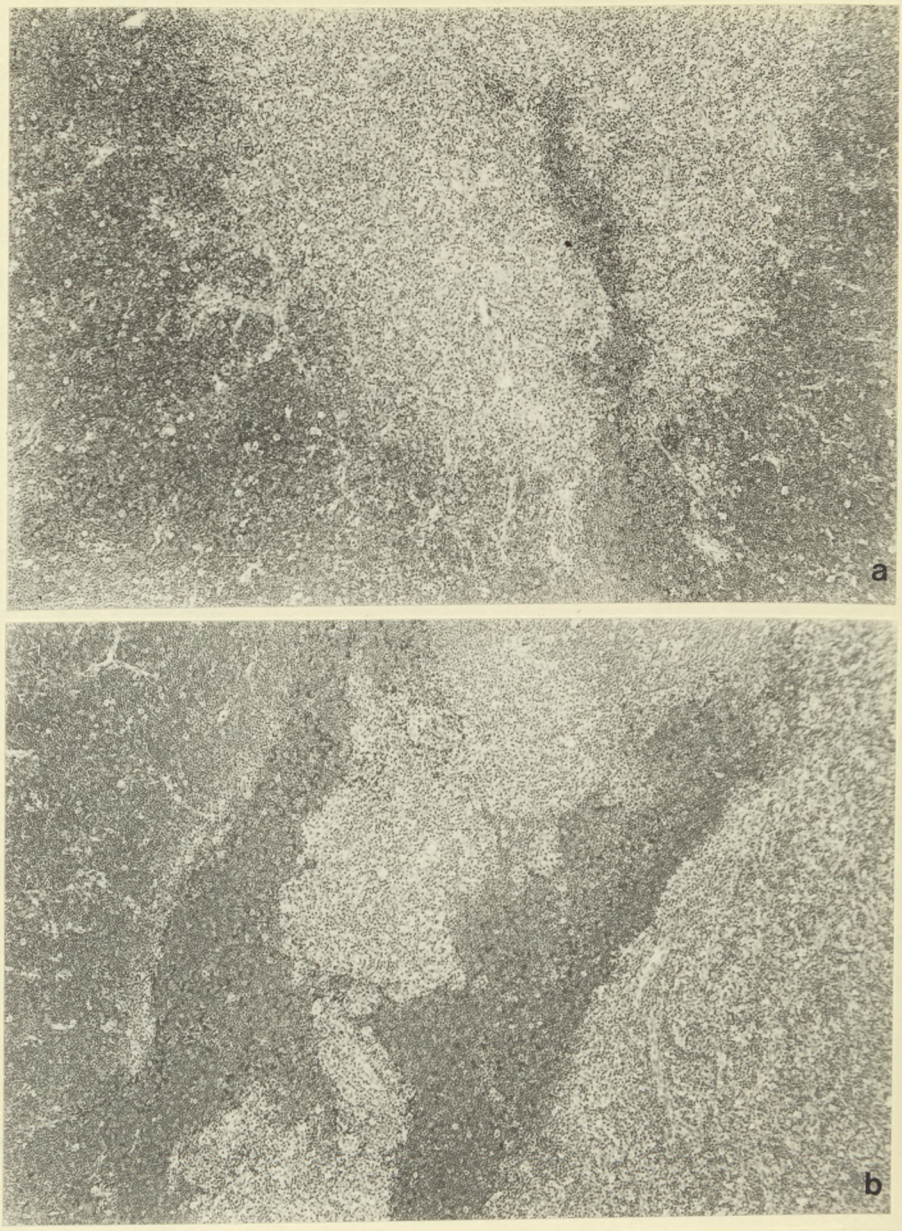


Figure: 3.7. Representative photographs of the histology of the thymus of (a) Pairfed and (b) Vitamin A deficient mice. H & E stain, X 70. There is no difference in structure apparent between vitamin A and pairfed groups.

medullary and cortical regions were found to be similar in all groups, although it is difficult to compare the cortical or medullary areas between different sections. In practice it is not possible to sample the same relative area of thymus for histological examination for all groups of mice. As we had seen a relative fall in thymus weight in deficient animals as compared to controls, we were expecting the deficient thymus to be less cellular. However we could not reliably compare the area of the medulla or cortex of the deficient animals with the controls. Krishnan et al (1974) have claimed that the vitamin A deficient thymic cortex is almost completely depleted of lymphocytes but that the medulla appears unaffected. This is not evident in our experimental animals.

3.3.5.3. Lymph node:

The histology of the mesenteric lymph nodes in all groups of mice was examined at different time intervals. The B-cell areas of the lymph node of all the groups were normal and contained variable numbers of lymphoid follicles. The T-cell areas of all groups appeared normal.

3.3.5.4. Gut:

The histology of the small intestine and Peyer's patches of all groups was examined. Photographs of representative areas of gut from different groups are shown in figure: 3.8. The villi of all mice examined appeared normal and intact. The villus length in all groups was similar. The structure of the Peyer's patches did not differ between control and test animals. Deluca et al (1969) have shown the histological appearance of the small intestine to be normal in vitamin A deficient rats as compared to pairfed animals with the exception of a reduction in mucous secreting goblet cells.

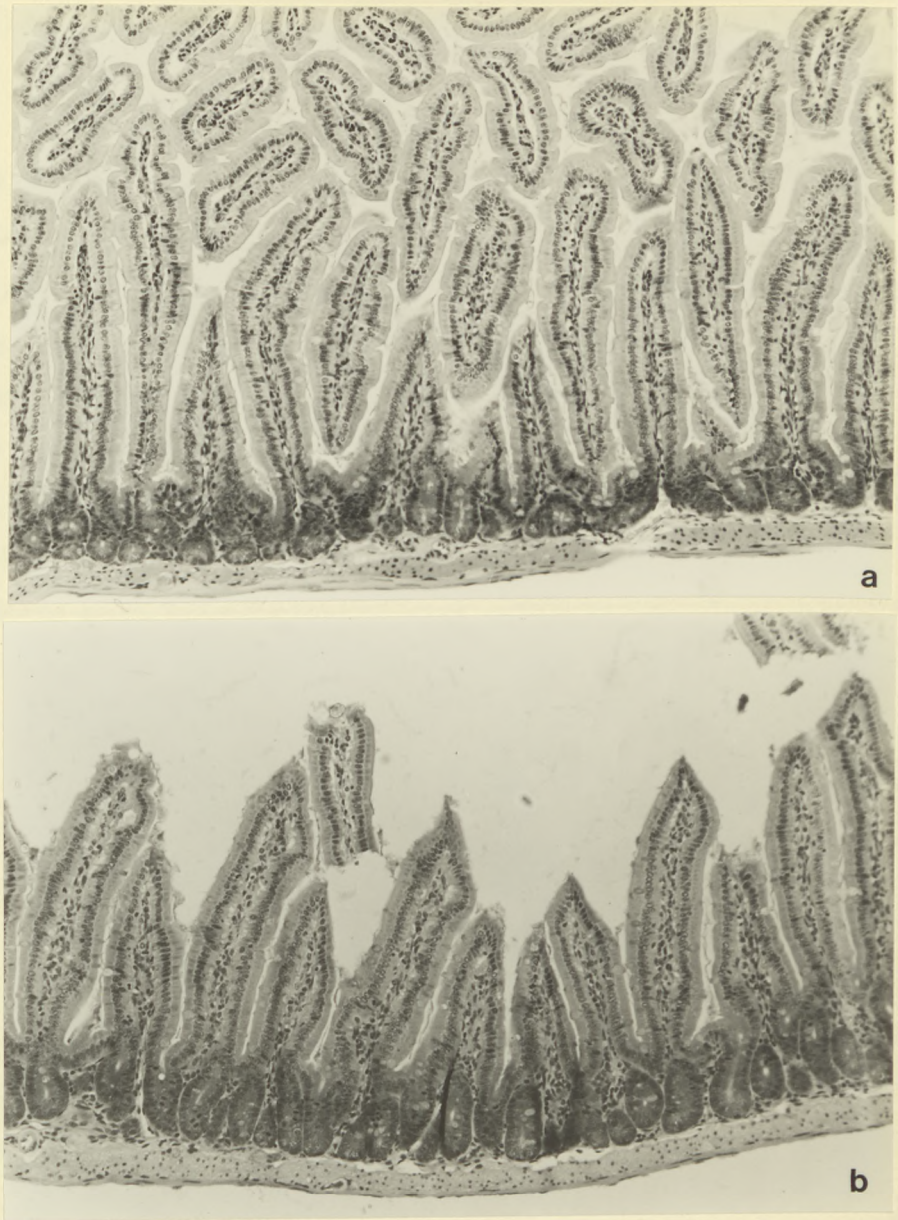


Figure: 3.8. Representative photographs of the histology of the small intestine (middle portion) of (a) Pairfed and (b) Vitamin A deficient mice. H & E stain, X 112. There is no difference apparent in the villus structure in either case.

3.4. Conclusions:

The growth of vitamin A deficient animals was normal for approximately 4-5 weeks on the experimental diet. During this period they utilized endogenous vitamin A from liver stores. The liver store of vitamin A was nearly exhausted by 6 weeks of feeding. At this point the growth of vitamin A deficient animals began to diverge from that of control animals. Our result confirms the finding of Zile et al (1979) who have shown a reduced overall weight gain in vitamin A deficient animals when compared with controls. Their results showed that the decreased growth rate was evident after 21 days of feeding and became significant after 30 days of feeding.

From the experiments described it is clear that the growth of vitamin A deficient animals ceased and they entered a weight plateau stage at about 9 weeks on the experimental diet. Also at 9 weeks the liver stores of vitamin A of the vitamin A deficient animals were completely exhausted and serum levels of vitamin A were about 85% lower than the control and pairfed animals. Liver is the organ that stores approximately 90% of the body vitamin A and supplies vitamin A to the circulation by a homeostatic mechanism (Underwood et al.,1979) to meet the needs of the tissues for normal function. Therefore, on the basis of the liver and serum vitamin A levels we considered our animals to be at an early stage of vitamin A deficiency at 9 weeks. We continued to feed the deficient diet for a further 3 weeks, during which the body weight remained constant. It has been suggested that vitamin A deficiency is associated with a constant body weight and any fall in body weight is due to secondary infection (Bieri,1969).

We observed that the weight of the liver of vitamin A deficient animals was lower than that of the controls but not of the pairfed animals. However, when liver weight was calculated in terms of percentage of body weight, their was no significant difference observed between deficient and the two control groups. Therefore, lack of vitamin A alone does not affect the liver weight. Others have also

reported normal hepatic function in vitamin A deficiency (Zile et al.,1979) and absence of histological changes in deficient animals (Moore,1957).

In general we observed that vitamin A deficiency has an effect on body weight and growth rate. Some organs, especially the thymus are also affected, but no gross histological changes were seen. We did not see any other clinical sign of vitamin A deficiency in these animals.

Since the deficient animals did not show any weight loss and there were no histological changes in the lymphoid organs, we concluded that they had not contracted any intercurrent infections. Therefore, this experimental design provided us with a suitable vitamin A deficient mouse model for the investigation of the immune responsiveness to rotavirus infection.

CHAPTER FOUR

ROTAVIRUS INFECTION AND IMMUNIZATION

CHAPTER FOUR

4.1. Introduction:

Diarrhoeal diseases are among the most common infections of infants and young children throughout the world, and they assume a special significance in less developed countries where they constitute a major cause of mortality among the young. Viruses are a major cause of diarrhoeal episodes in infants and young children in both developed and underdeveloped countries (W.H.O. Scientific Working Group, 1980). Rotavirus is now widely recognized as one of the major etiologic agents of episodic gastroenteritis of infants and young children in most areas of the world (Barnett, 1983; Cukor and Blacklow, 1984; Kapikian et al., 1976; Kapikian et al., 1980).

The discovery in 1973 of the 70 nm human rotavirus and its association with gastroenteritis of infants and young children represents a major advance in elucidating the cause of acute infectious non-bacterial gastroenteritis (Bishop et al., 1973). Although the human rotaviruses were discovered in 1973, Adams and Kraft in 1963, described virus like particles in intestinal tissue of mice infected with the agent of epizootic diarrhoea of infant mice (EDIM, Adams and Kraft, 1963).

In the present section, experiments were designed to investigate the potency of EDIM rotavirus. The procedure for infection and the development of immunity to the EDIM strain of rotavirus are described.

4.2. Experimental procedure:

4.2.1. Potency test for the EDIM rotavirus:

4.2.1.1. Infection in rotavirus free infant mice:

7 day old male CD1 outbred mice (rotavirus free) were obtained from Charles River, U.K. Animals were housed with their mothers in a plastic cage in the isolation room. The room temperature was 22±2 °C, humidity 50.0 % and there was a 12 hour lighting schedule. These infant mice were infected with 30 µl/mouse of EDIM rotavirus (containing $10^{4.5}ID_{50}$) by oral dosing. At 48 hours post-dosing all animals were killed and the histology of the small intestine (middle portion) was examined by H & E staining as described in section-2.5.

4.2.1.2. Infection in conventional infant mice:

7 day old male Porton mice (conventional) were housed with their mother in the isolation room. The room temprature was 22±2 °C, humidity 50.0 % with a 12 hour lighting schedule. These infant mice were infected with 30 µl/mouse of EDIM rotavirus (containing $10^{4.5}ID_{50}$) by oral dosing. At 7 days post-dosing all animals were killed and the histology of the small intestine (middle portion) was examined by H & E staining as described in section-2.5.

4.2.2. Test for swallowing of rotavirus:

4.2.2.1. By using blue food colouring:

Two male Porton mice (3 months old) were housed individually in a plastic cage with sawdust as a bedding. Each mouse was fed orally with 200.0 µl of blue food colouring solution using the dosing needle

employed for virus dosing. The animals were examined over the next 5 hours to identify whether there had been any discharge of food colouring from the mouth.

4.2.3. Test for the production of antibody:

3 month old male Porton mice were fed 30 µl/mouse of EDIM rotavirus orally. A number of animals were killed at 0, 2, 5, 9 days post dosing and blood samples were collected. The total circulating antibody level specific to rotavirus was measured by using a cell-ELISA method as described in section-2.8.1.

4.3. Results and discussions:

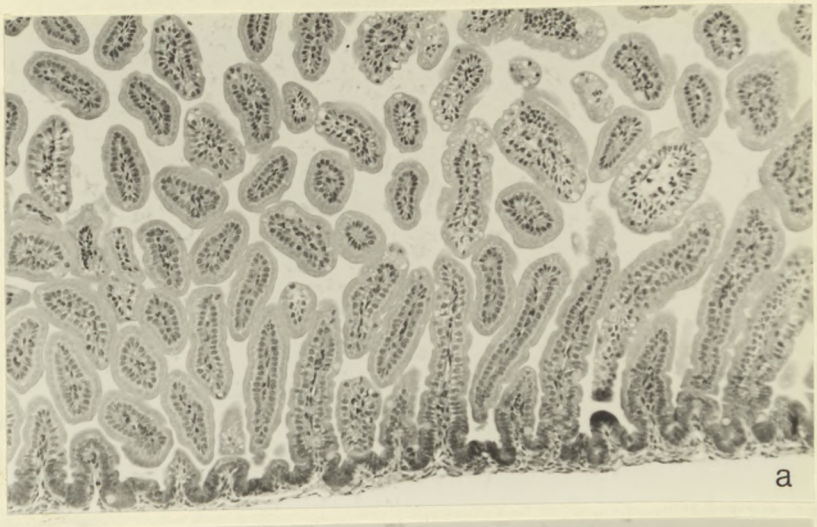
4.3.1. Potency test for the EDIM rotavirus:

4.3.1.1. Histology of the gut 48 hours post-dosing:

The histology of the small intestine of infected mice (rotavirus free) was examined after 48 hours of dosing under light microscopy and compared with control gut.

The non-infected control gut appeared normal and the villi were intact. On the other hand, the villi of the infected gut were swollen at the tip and narrow at the bottom. The tips of the villi appeared foamy with extensive vacuolation in their upper half. The epithelial cells were less compact than normal and there were many pyknotic nuclei. A representative photograph of the histology is shown in figure: 4.1. These observations indicate that the EDIM strain of mouse rotavirus was active and virulent. However, the animals in our study did not show any diarrhoea, but the histological changes are similar to those reported by Starkey et al (1986). A possible reason why we did not see diarrhoea might have been that the mothers cleaned the pups quickly.

Figure: 4.1. Representative photographs of the histology of the small intestine of infant mice. (a) Non-infected control (CD1 mice), (b) 48h post infection (CD1 mice), and (c) 7 days post infection (Porton mice). H & E stain, X 112.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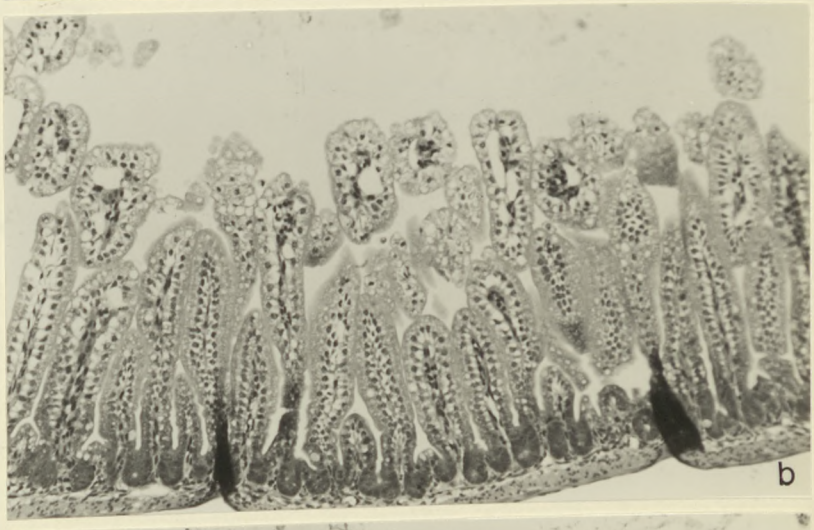
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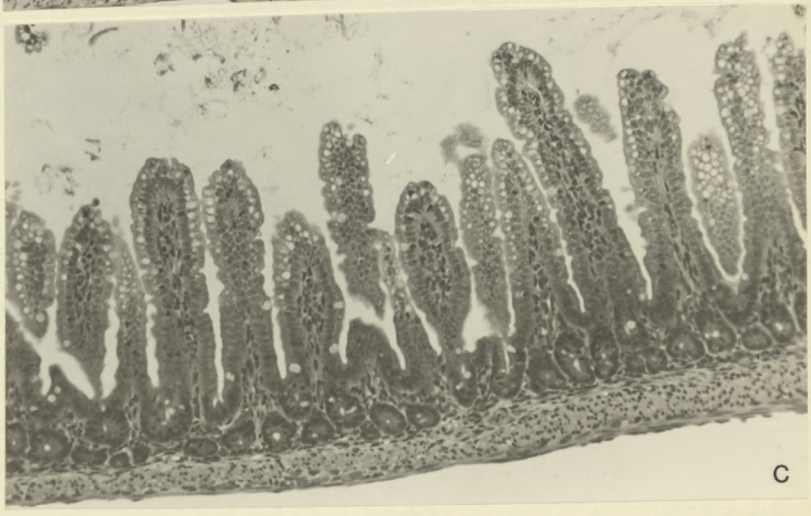
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4.3.1.2. Histology of the gut 7 days post-dosing:

We also infected the conventional infant mice to ascertain whether the EDIM rotavirus caused any histological damage. In these experiments we noted diarrhoea in 50 % animals after 48 hours of dosing. These animals were killed after 7 days post-dosing and the histology of the small intestine was examined under the light microscope. A representative photograph is shown in figure: 4.1. The tips of the villi appeared foamy and many vacuolations were observed within epithelial cells. The epithelial cells at the tips of the villi appeared necrotic. This experiment therefore, provides evidence that the EDIM rotavirus was virulent and was able to cause diarrhoea in conventional mice.

4.3.2. Test for the swallowing of antigen:

After the feeding of blue food colouring solution in water, we observed the animals each hour over the next 5 hours. No blue colour was observed either in the plastic cage or in the bedding. Further, we observed blue coloured feces in the bedding after some hours. This observation confirmed that the use of the dosing needle was an effective way of administering an oral dose of the virus.

4.3.3. Antibody production:

Antibody titres are shown in figure-4.2. At day 2, the antibody levels were similar to non-infected normal mice. Thereafter, the antibody titre increased progressively. At day 5, the antibody titre was 128 and at day 9, the titre was 256. These results show that oral challenge with rotavirus provokes an antibody response in conventional adult mice. Little and Shaddock (1982) have observed that seronegative infant mice infected orally with mouse rotavirus showed a substantial rise in serum antibody levels by 8 day of infection. They have also shown that in seropositive infant mice a rise in serum

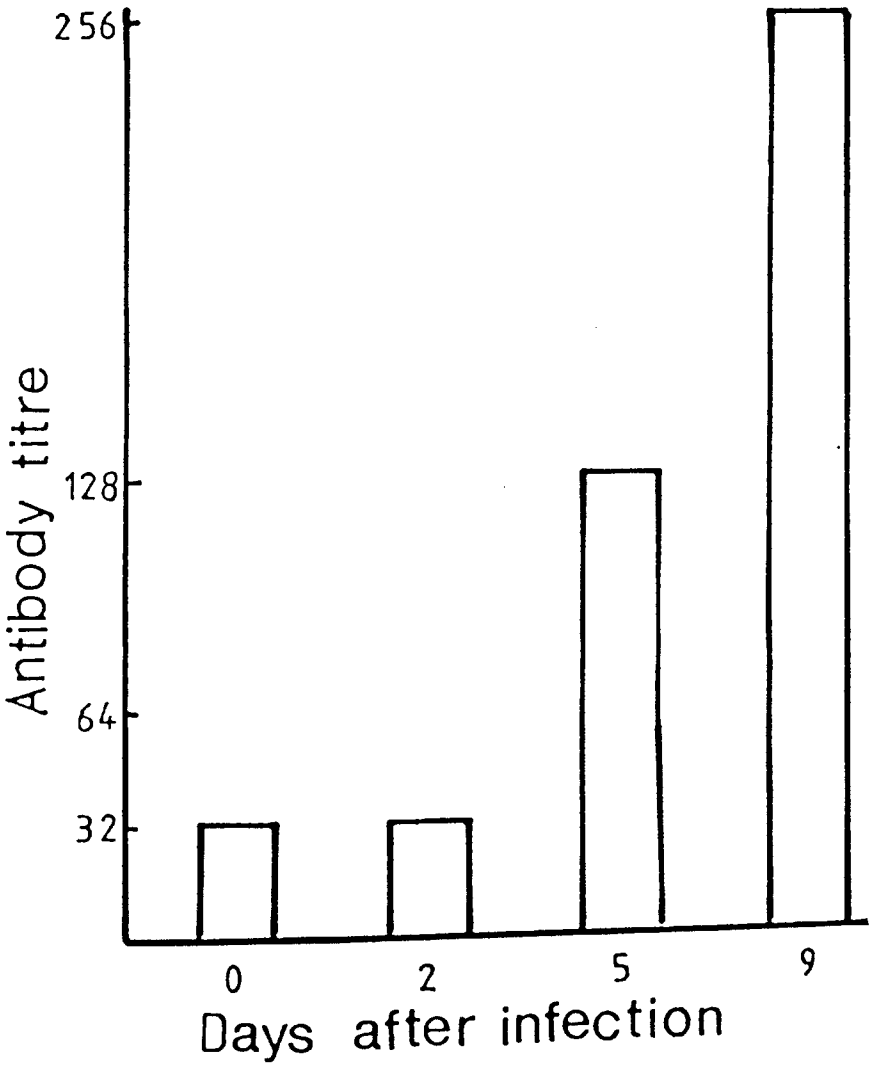


Figure: 4.2 A histogram showing antibody titre at different time intervals after an oral dose of EDIM rotavirus. Each bar indicates the mean of two animals.

antibody titre was evident as early as 96 hours post-dosing.

4.4. Conclusions:

From the above experiments it was confirmed that the EDIM strain of rotavirus used in this study was potent and virulent. Further, it was capable of causing histological damage. In these studies we examined the middle portion of the small intestine as this is the area most infected by EDIM rotavirus and in which abnormal villus architecture was reported by Starkey et al (1986). The procedure of enteral infection with a dosing needle was also satisfactory. Finally we also demonstrated that the EDIM rotavirus was able to provoke antibody production in adult conventional mice.

CHAPTER FIVE

EFFECT OF VITAMIN A DEFICIENCY ON THE ROTAVIRUS INFECTION

CHAPTER FIVE

5.1 Introduction :

Vitamin A deficiency has been shown to be associated with various infectious diseases. For instance, Nauss et al (1985a) have shown that experimental ocular Herpes simplex virus (HSV) infections were more severe in vitamin A deficient rats when compared with the pairfed controls. Krishnan et al (1976) have reported that parasitemia increased at a faster rate in vitamin A deficient rats, after infection with the malarial parasite Plasmodium berghei, compared with control and pairfed animals. Sriramachari and Gopalan (1958) have also shown in rats that vitamin A deficiency leads to a relatively more severe tuberculosis infection, as compared to controls. Decreased resistance to infection with Newcastle disease virus (NDV) has been reported in vitamin A deficient chicks following intranasal inoculation (Bang and Foard, 1971). This group also showed increased susceptibility of vitamin A deficient chicks to influenza virus.

Although it has been recognized for years that vitamin A deficiency predisposes the host to infection, the relevant information following oral challenge is limited (Darip et al, 1979). Indeed, to the best of our knowledge, no reports are available so far on gut immunity to enteric virus in vitamin A deficient animals. Therefore, the present study was conducted to investigate the effect of vitamin A deficiency on the immune response against mouse rotavirus infection.

5.2. Experimental procedure:

The study protocol is presented in figure :5.1 and the parameters measured in figure :5.2. The routine procedures have been described in chapter-2.

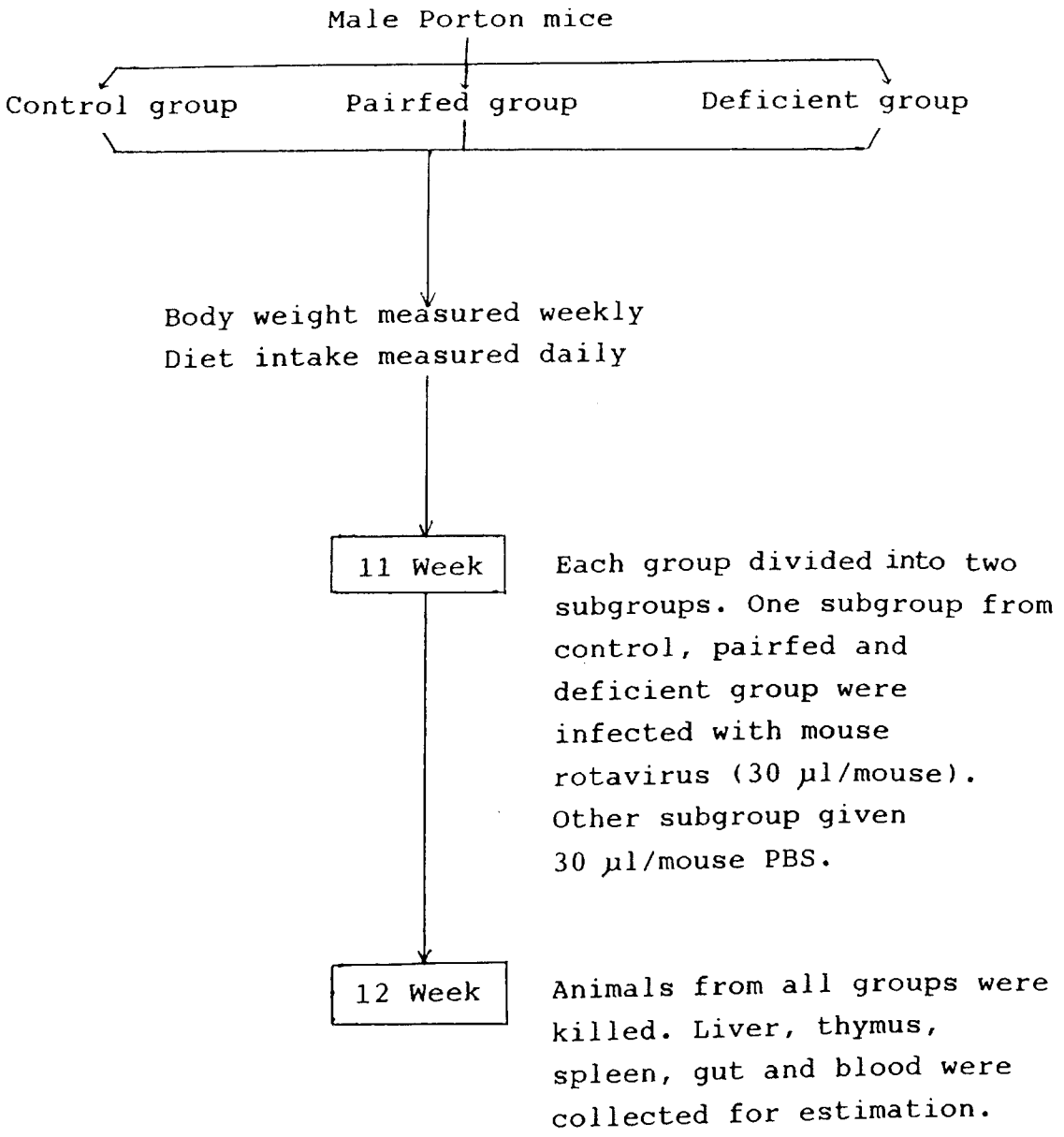
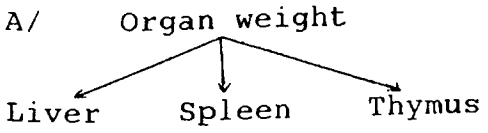
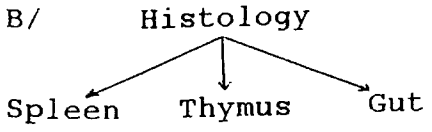
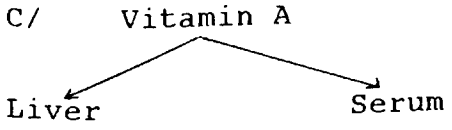
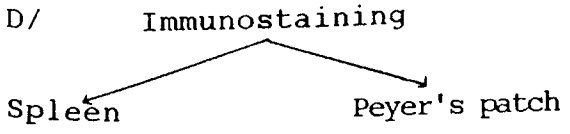


Figure:5.1 Scheme of the procedure for the study of the interaction of vitamin A deficiency and rotavirus infection.









F/ Intestinal goblet cell count.

Figure: 5.2 Outline of the parameters measured in the investigation of the interaction of vitamin A deficiency and rotavirus infection.

5.2.1. Procedure for rotavirus infection :

Male Porton mice, 19-21 days old, were initially weight matched and divided into three groups, as control, pairfed and vitamin A deficient. They were supplied with their respective diet as described in chapter-2.2. The weights of the animals were taken weekly and food consumption measured daily.

All animals were kept on their respective diet for 11 weeks and then each group was divided into two subgroups. Animals from one subgroup were infected with 30 µl/mouse of EDIM rotavirus by oral dosing and termed "infected". Animals from the other subgroup were dosed with phosphate buffer saline (30 µl/mouse) and termed "non-infected".

One week post-dosing, the animals were bled by heart puncture and blood samples were allowed to stand at room temperature for one hour to allow clot formation. After clotting, the blood was centrifuged and the clear serum removed with a pasteur pipette. The serum samples were stored in ependorff tubes at -20°C until analysed. Immediately after the blood was collected, the carcase of the animals was cut open and the liver, spleen and thymus excised and weighed. Liver was stored at -20°C until analysed for the vitamin A content. Spleen, thymus, a representative portion of small intestine and duodenum were fixed in 10% formalin buffered solution for histological analysis.

5.2.2. Determination of vitamin A:

5.2.2.1. Liver:

Liver vitamin A was determined by the method of Bayfield (1975) with modifications (Section-2.3).

5.2.2.2. Serum:

Serum vitamin A was estimated by the spectrophotometric method of Bradley and Hornbeck (1973) with slight modification (Section-2.4.1).

5.2.3. Histological study:

The histology of the spleen, thymus, and gut was examined by the method described in section-2.5.

5.2.4. Mucin staining:

Goblet cells and the mucins of duodenal villi were stained by the method of Cook (1962) described in section-2.6.

5.2.5. B and T cell staining:

B and T cells of spleen and Peyer's patches were stained by an immunoperoxidase technique described in section-2.7.3. and 2.7.4.

5.2.6. Statistical analysis:

Results are expressed as the mean and standard error of the mean. Differences between groups were evaluated statistically using Student's "t" test.

Differences in mean values were considered significant when $P<0.05$.

5.3. Results and discussions:

5.3.1. Body weights:

The body weights of all groups throughout the experiment are shown in figure-5.3. The growth pattern for all the groups was similar

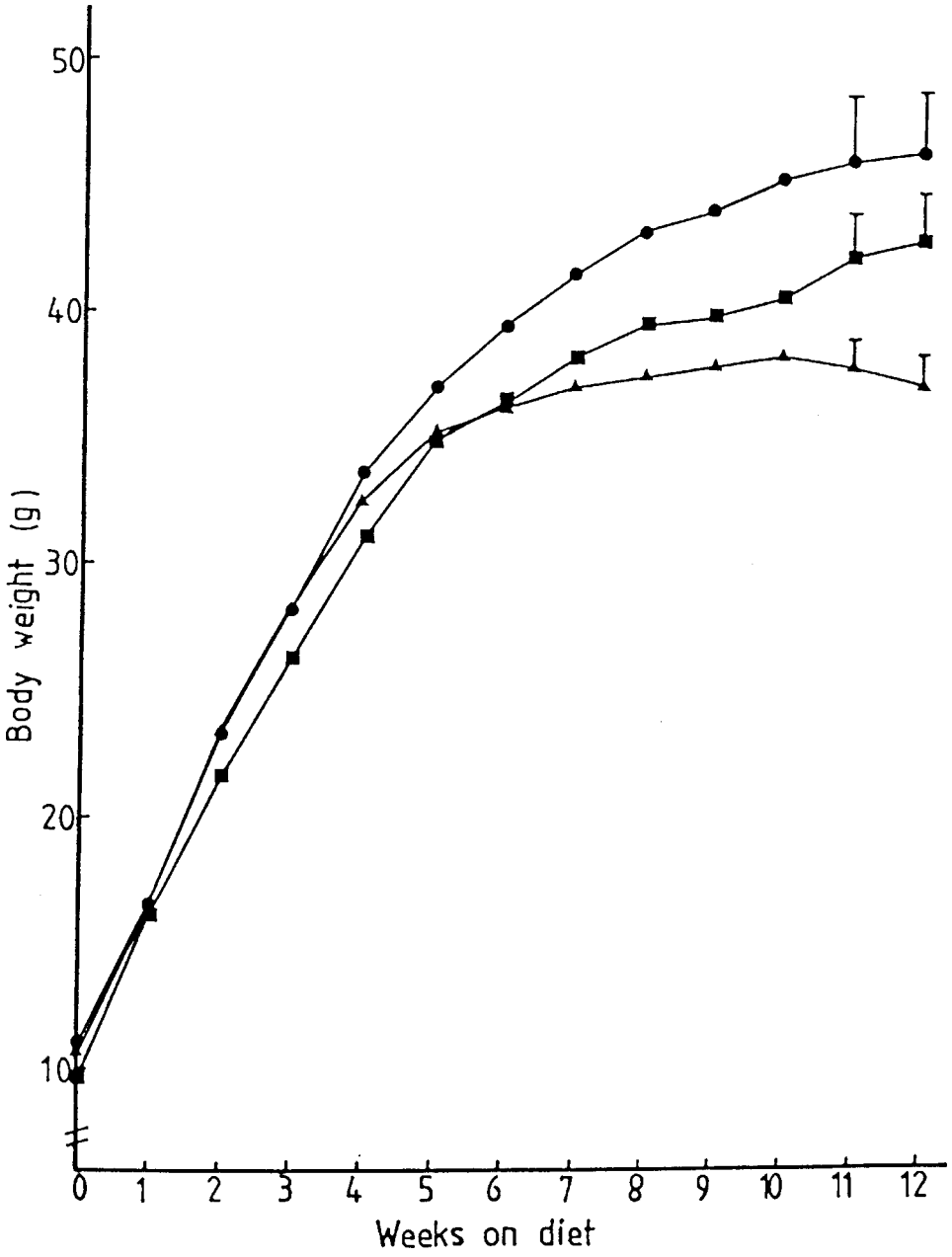


Figure:5.3 Body weight gain of different groups of animals during the experiment. Control (●——●), pairfed (■——■) and vitamin A deficient (▲——▲) group. Each point indicates the mean of 8 animals. The 11 & 12 week time points display the mean±SEM.

to the experiments described in chapter-3. We noted that the intake of these groups was slightly lower than the corresponding previous groups. The deficient animals in the present study had eaten less diet compared with controls. The two studies were carried out at a 3 month time interval and used animals from a different colony. The first study was performed in the Boldrewood animal house, Southampton University and the second study in the Southampton General Hospital animal house. Therefore, these changes may account for the difference in body weight of the animals between the studies.

The body weights of vitamin A deficient animals were significantly lower than those of control ($P<0.001$) and pairfed ($P<0.01$) animals after 11 weeks of feeding, when the animals were infected. There was no significant difference between pairfed and control groups.

5.3.2. Diet intake:

The daily dietary intake of the control and vitamin A deficient groups are shown in figure :5.4. The intake of vitamin A deficient animals was slightly lower than the control after 9 weeks. There was no change observed in the intake after rotavirus infection in either vitamin A deficient or control animals.

5.3.3. Organ weights:

5.3.3.1. Liver:

Total liver weights for the non-infected and infected groups are shown in table :5.1. The total liver weight of vitamin A deficient animals, both infected and non-infected, were significantly lower than those of the corresponding control groups ($P<0.05$). The liver weights of the vitamin A deficient infected group were significantly lower than the pairfed group ($P<0.05$), but this was not the case with the non-infected group. This discrepancy probably reflects the

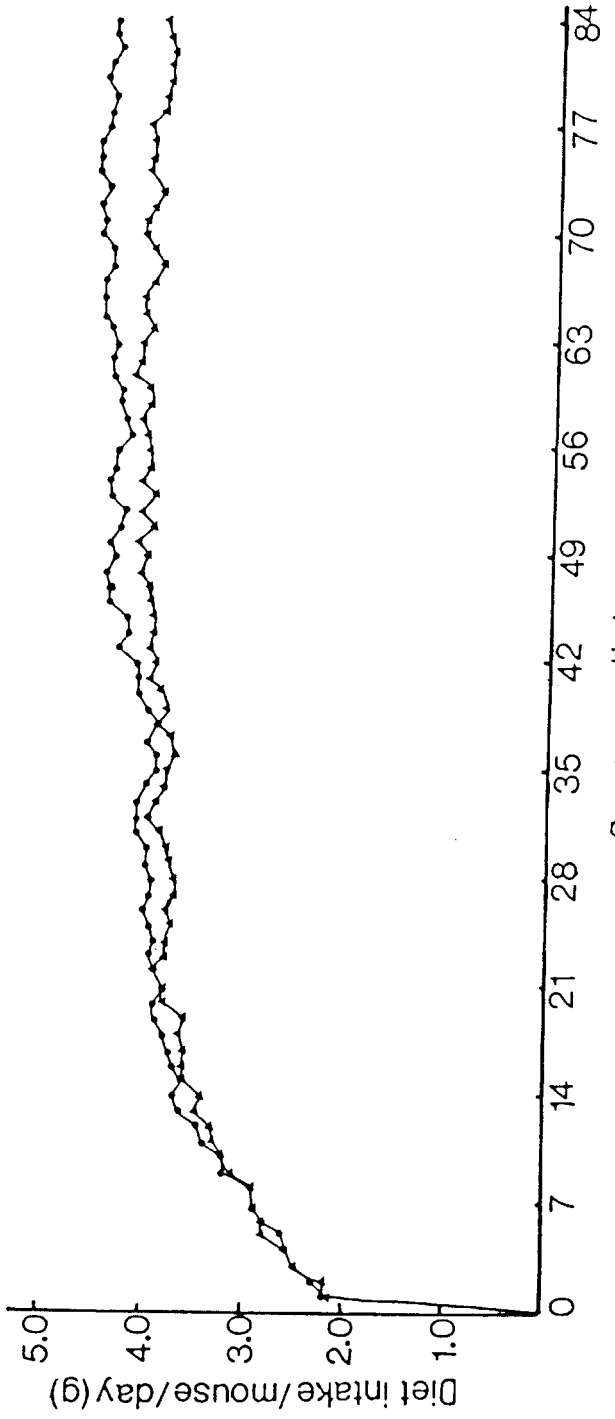


Figure: 5.4. Daily diet intake of the control ad libitum (●—●) and vitamin A deficient animals (▲—▲) throughout the study.

Table :5.1

Effect of vitamin A deficiency and rotavirus infection on the total liver weight (Mean±SEM)

Group	Infection (5) g	Non-infection (3) g
Control	1.80±0.1	1.80±0.08
Pairfed	1.72±0.1	1.51±0.09
Deficient	1.43±0.06*	1.49±0.03 **

Figures in the parentheses indicate sample size.

*Significantly different from corresponding control and pairfed groups by "t" test, ($P<0.05$).

**Significantly different from corresponding control group by "t" test, ($P<0.05$).

smaller number of animals used in the non-infected group. There was no significant change in liver weight observed due to infection. Takagi and Nakano (1983) showed that the liver weights of both non-immunized and immunized vitamin A deficient animals were significantly lower than those of the corresponding control animals. This observation supports our own findings. Our results indicate that the vitamin A deficiency when associated with low food intake interferes with the weight of the liver. Rotavirus infection does not have any effect on liver size.

A histogram of relative liver weights (as % of body weight) is presented in figure: 5.5. There was no significant difference observed between vitamin A deficient animals and the corresponding control and pairfed groups. Further, there was no significant difference found due to infection.

5.3.3.2. Spleen:

Total spleen weights of all groups are shown in table:5.2. There was no significant change observed between the total spleen weight of non-infected vitamin A deficient animals and the corresponding control and pairfed animals. The total spleen weights of infected vitamin A deficient animals, in contrast, were significantly higher than those of infected controls ($P<0.01$) and pairfed animals ($P<0.01$). There was no significant change observed between the pairfed and control groups in either the non-infected or infected animals. This result indicates that vitamin A deficiency, either alone, or when associated with low food intake does not significantly alter the weight of the spleen, although spleens in vitamin A deficient animals had a tendency to be of slightly higher weight or larger in size. The observation that the spleens of vitamin A deficient infected animals were significantly higher than those of the control and pairfed groups, suggests that in the deficient animals there was a systemic response following rotavirus infection.

A histogram for relative spleen weights (spleen weight as %

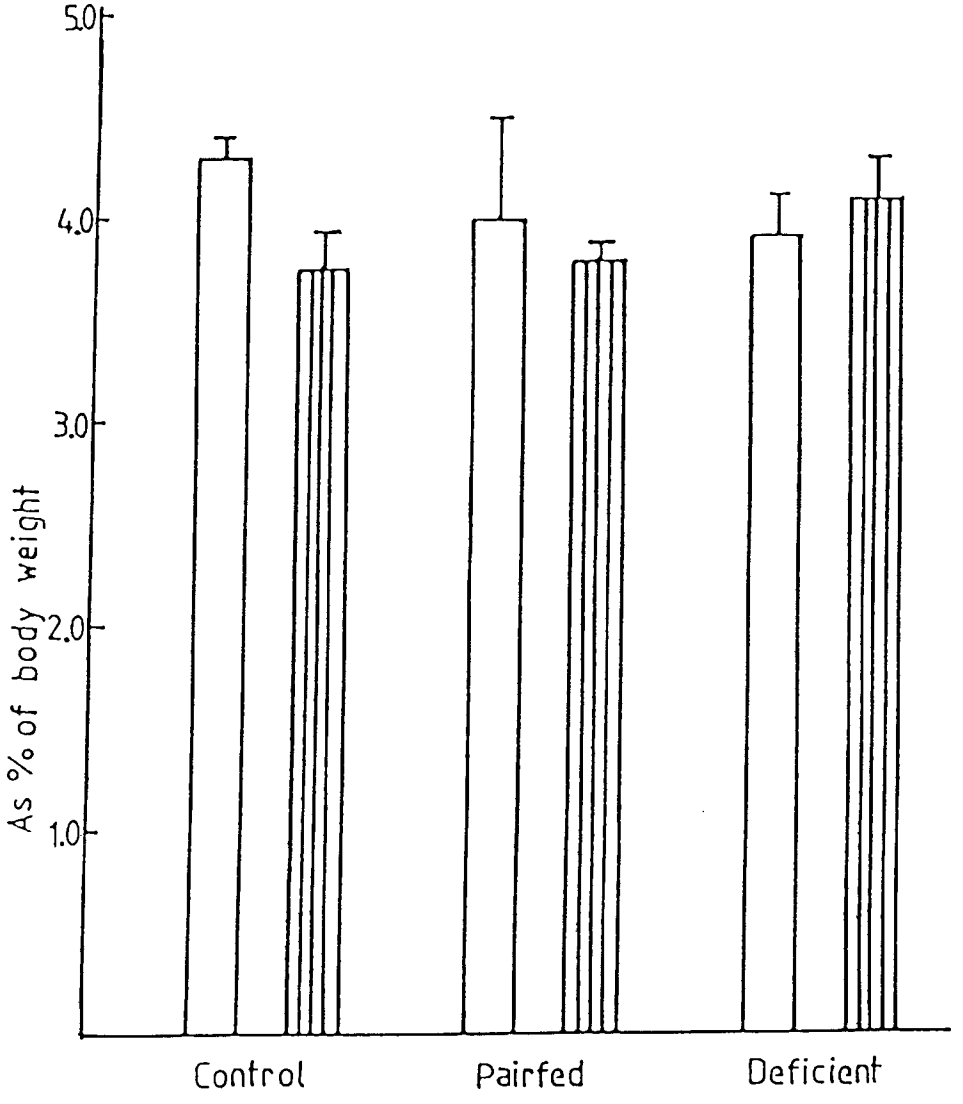


Figure: 5.5. A histogram showing the relative liver weights of different experimental groups. Infected (▥) and Non-infected (□).

Table :5.2

Effect of vitamin A deficiency and rotavirus infection on the total spleen weight (Mean±SEM)

Group	infected (5) mg	non-infected (3) mg
Control	100.8±6.5	101.0±12.7
Pairfed	101.6±8.6	111.5±7.8
Deficient	158.0±12.8*	138.0±10.2

Figures in the parentheses indicate sample size.

*Significantly different from corresponding control and pairfed groups by "t" test, ($P<0.01$).

of body weight) is presented in figure :5.6. The results for relative spleen weight are similar to the results for total spleen weight. Takagi and Nakano (1983) have shown significantly lower spleen weight in vitamin A deficient rats immunized with sheep RBC compared with controls. But their animals were immunized by intramuscular injection and therefore, both control and the deficient group showed a systemic response, whereas in our experiment we presume that a breakdown in the mucosal barrier may have allowed greater access of virus to the systemic immune system.

5.3.3.3. Thymus:

Total thymus weights of all groups are shown in table :5.3. The total thymus weight of vitamin A deficient animals in both the infected and non-infected groups were significantly lower than those of the corresponding control($P<0.001$) and pairfed groups ($P<0.001$). No significant changes were found between pairfed and control animals. Again, the thymus weights in vitamin A deficient infected animals were found to be significantly lower than in the non-infected vitamin A deficient group ($P<0.05$). The results indicate that vitamin A deficiency either alone or in conjunction with low food intake slows the growth of the thymus. Rotavirus infection associated with vitamin A deficiency leads to further weight loss.

A histogram of relative thymus weights (as % of body weight) for all the groups is presented in figure :5.7. The results for relative thymus weight are similar to those for total thymus weight. The relative thymus weights of infected vitamin A deficient animals were significantly lower than those of the non-infected vitamin A deficient group. The results indicate that vitamin A deficiency alone or when associated with low food intake can also lead to a reduction of relative thymus weight. Again, vitamin A deficiency when associated with rotavirus infection can cause further reduction in the size of the thymus.

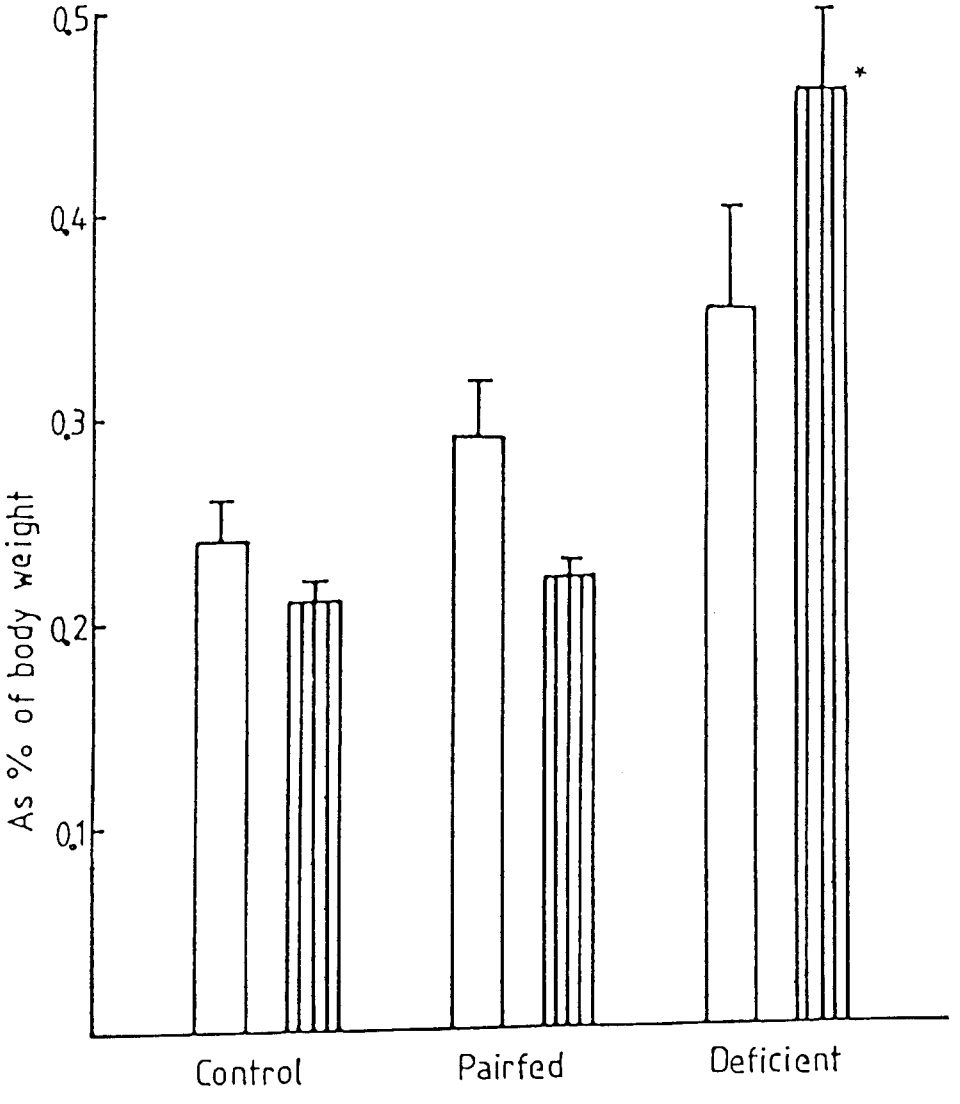


Figure: 5.6. A histogram showing the relative spleen weights of different experimental groups. Infected (▥) and Non-infected (□). * Significantly different from corresponding control and pairfed animals by "t" test ($P<0.001$).

Table :5.3

Effect of vitamin A deficiency and rotavirus infection on the total thymus weight (Mean±SEM)

Group	Infected (5) mg	Non-infected (3) mg
Control	46.0±3.7	47.3±2.2
Pairfed	49.4±3.7	41.5±2.6
Deficient	24.7±2.0**	32.2±1.7*

Figures in the parentheses indicate sample size.

Significantly different from corresponding control and pairfed group by "t" test. *(P<0.05) **(P<0.001).

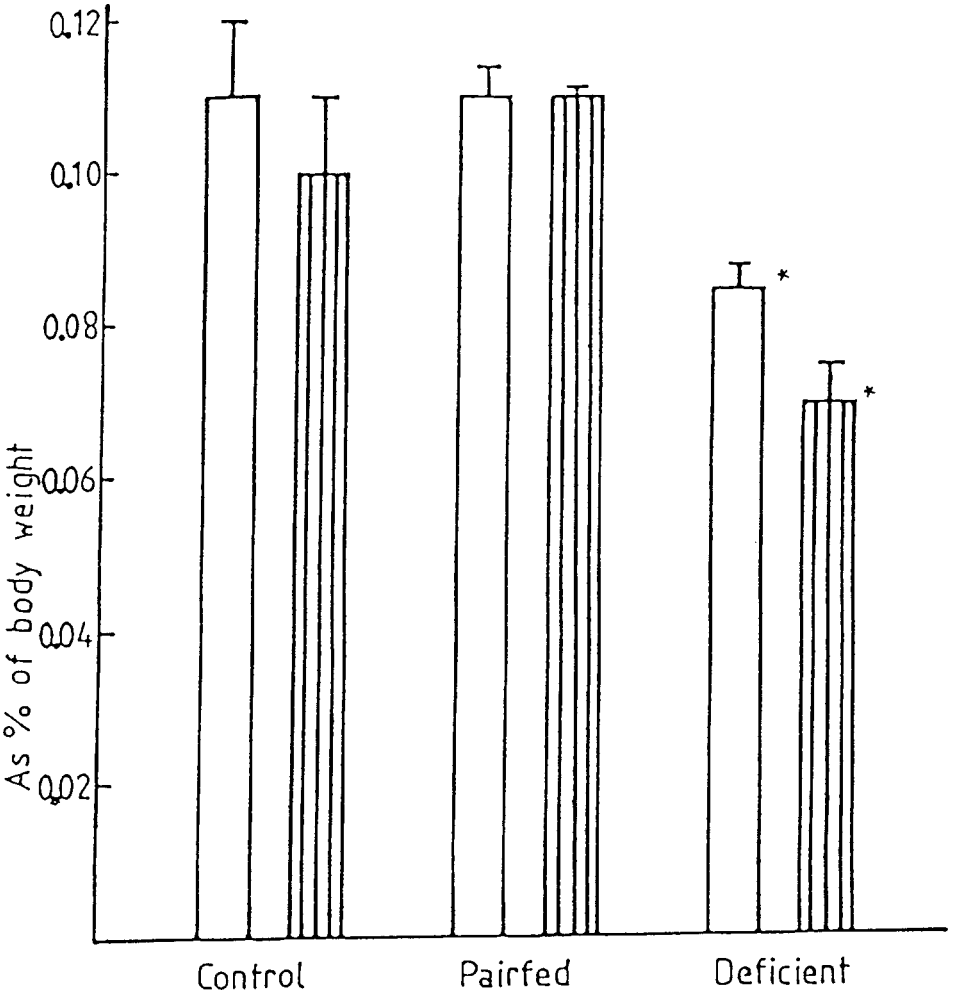


Figure: 5.7. A histogram presenting the relative thymus weights of different experimental groups. Infected (▨) and non-infected (□). * Significantly different from the corresponding control and pairfed group by "t" test (P value at least <0.05)

5.3.4. Vitamin A levels:

5.3.4.1. Liver vitamin A :

Liver vitamin A levels for both infected and non-infected groups are shown in table :5.4. Liver vitamin A levels for the vitamin A deficient group were significantly lower than those of corresponding control and pairfed groups ($P<0.001$). There was no significant change observed between the pairfed and control groups. Moreover, there was no significant difference between infected and noninfected liver vitamin A stores.

5.3.4.2. Serum vitamin A :

Serum vitamin A levels for all groups of non-infected mice are shown in table :5.5. Serum vitamin A levels of vitamin A deficient animals were significantly lower than those of control and pairfed animals ($P<0.001$). But there was no significant difference observed between pairfed and control groups. We did not measure the serum vitamin A levels of the infected group as this would have reduced the volume of serum available for the measurement of serum antibody levels.

5.3.5. Histological studies:

5.3.5.1. Spleen:

The histology of spleen of both non-infected and infected groups was examined by light microscopy. Both the white pulp and red pulp areas of non-infected vitamin A deficient animals were similar to those of control and pairfed animals. The results of this study are similar to those obtained with non-infected animals in the previous study.

Table:5.4

Effect of vitamin A deficiency and rotavirus infection on the liver vitamin A (Mean±SEM)

Group	Infected (5) µg/Liver	Non-infected (3) µg/Liver
Control	250.8±12.9	237.9±8.7
Pairfed	220.8±12.8	215.7±8.0
Deficient	0.5±0.1*	0.5±0.1*

Figure in the parentheses indicate sample size.

*Significantly different from control and pairfed group by "t" test ($P<0.001$).

Table: 5.5

Effect of vitamin A deficiency and rotavirus infection on the serum vitamin A (Mean±SEM)

Group	Non-infected (3) µg/100ml
Control	43.5±3.3
Pairfed	40.6±2.1
Deficient	4.6±0.8*

Figure in parentheses indicate sample size.

*Significantly different from control and pairfed group by "t" test ($P<0.001$).

The red pulp of infected vitamin A deficient animals could not be distinguished histologically from control and pairfed animals. When examined by eye under the light microscope it appeared that the infected vitamin A deficient spleen had more follicles than in the other groups. Representative photographs of the spleen of vitamin A deficient and pairfed mice are presented in figure :5.8. The histology of the infected vitamin A deficient group correlates well with spleen weight or relative spleen weight, and supports our contention that infected, vitamin A deficient animals may be undergoing a systemic response to either rotavirus or other infective agents.

5.3.5.2. Thymus:

The histology of the thymus for all groups was examined by light microscopy. The cortex and the medulla of vitamin A deficient animals were similar in appearance to those of control and pairfed groups. There were no changes observed between non-infected and the corresponding infected groups. Although the total and relative thymus weight were reduced both by vitamin A deficiency and rotavirus infection we observed no obvious histological changes in the thymic cortex or medulla. Changes in the relative areas of the thymic cortex and medulla may explain the low weight of thymus in vitamin A deficient animals. However, it was not possible to examine equivalent sections of thymus from different animals and therefore, these observations have to be interpreted with caution.

5.3.5.3. Gut:

The histology of the small intestine was examined by light microscopy. The villi and Peyer's patches of non-infected vitamin A deficient animals were similar to those of control and pairfed animals. The results of these non-infected groups were similar to those seen in previous experiments.

Significant changes in the villi of infected vitamin A

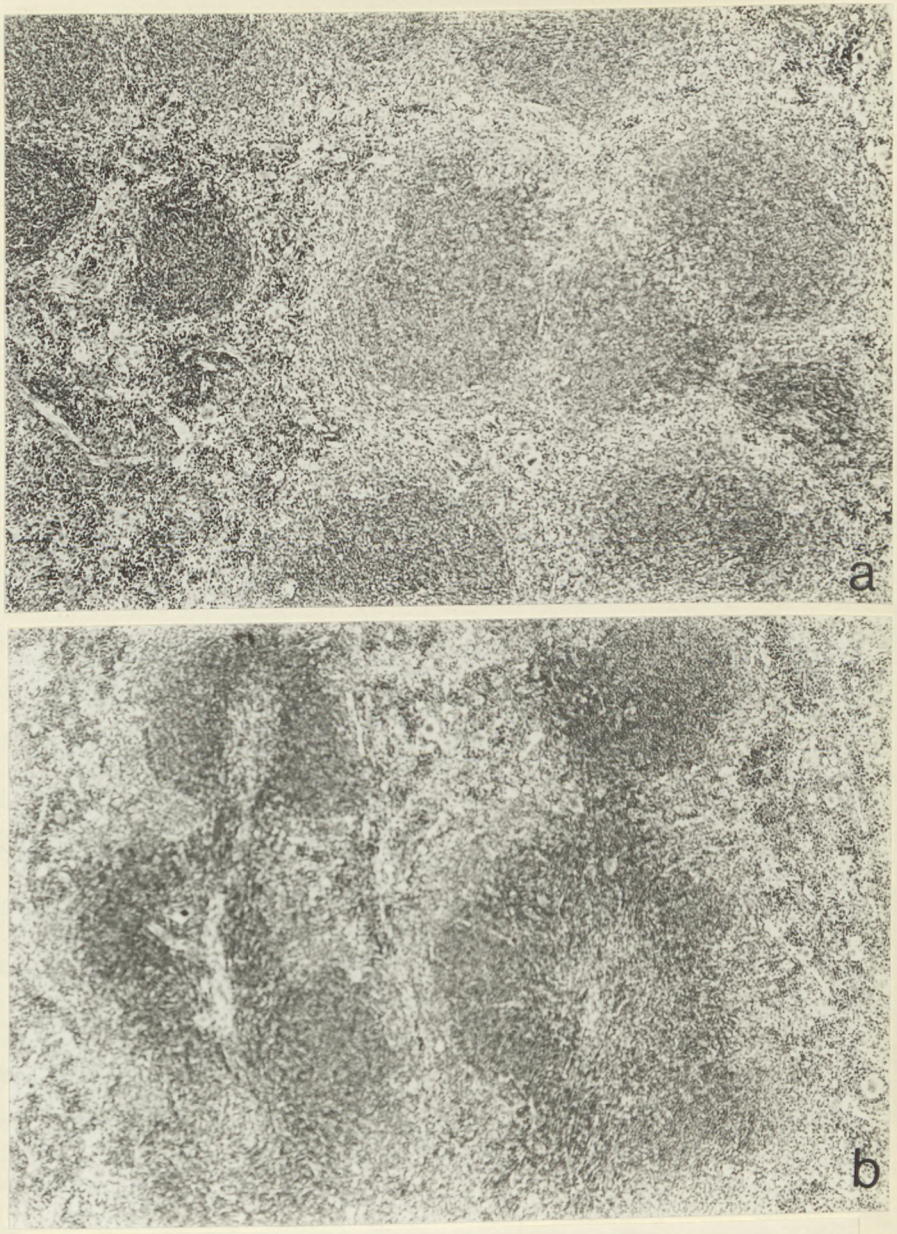


Figure: 5.8. Representative photographs of the histology of infected spleens from (a) pairfed and (b) vitamin A deficient mice. H & E stain, X 70.

deficient animals were observed compared to control and pairfed animals. The tips of the villi from the infected vitamin A deficient animals were almost destroyed whereas the villi of the control and pairfed animals were intact. In fact, we observed that 4 out of 5 vitamin A deficient animals had destroyed villus tips and the lamina propria of the villus core was exposed to the lumen of the small intestine. One vitamin A deficient animal had normal villus tips. On the other hand, in the case of the control and pairfed groups, 4 animals in each group appeared to have perfectly normal villus tips and, regular shaped villi, similar to non-infected animals. One animal from the control and one from the pairfed group showed slight disarrangement of the tips of the villi, however damage was less severe than was seen in the deficient animals. Therefore, the results indicate that the villi of the small intestine of vitamin A deficient animals showed major pathological changes following rotavirus infection. Rotavirus infection has been reported to affect the upper two-thirds of the intestinal villus in infant mice (Starkey et al.,1986) and vitamin A deficiency can also lead to changes in the epithelium of the small intestine (Deluca et al.,1969). These two observations are consistant with the pattern of destructive changes seen in the villi of infected animals in our experiments. Representative photographs are presented in figure :5.9. Photographs of the control animals are not shown as their histological appearance was similar to that of the pairfed animals.

The Peyer's patches of the vitamin A deficient animals appeared larger than those of control and pairfed animals irrespective of infection. No difference were noted between the control and the pairfed groups.

5.3.6. Non-specific immunity:

In order to investigate the effect of vitamin A deficiency either alone or in association with rotavirus infection on non-specific immunity, we enumerated the mucus secreting goblet cells

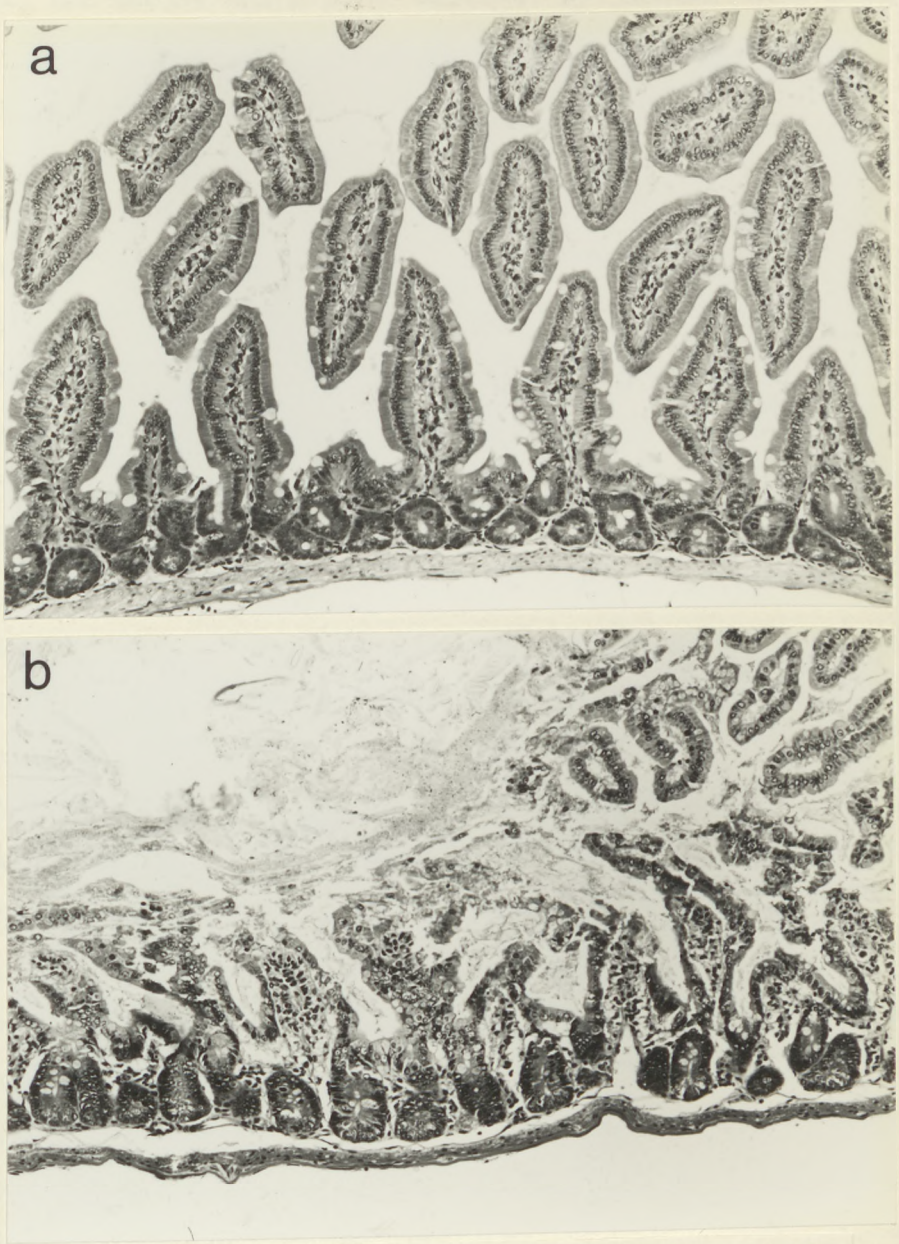


Figure: 5.9. Representative photographs of the histology of the infected gut (small intestine) of (a) pairfed and (b) vitamin A deficient mice. H & E stain, X 112.

of the small intestine of both infected and non-infected animals. First, the goblet cells were stained by alcian blue-periodic acid Schiff's reagent (AB-PAS), which stained all the acid and neutral mucin producing goblet cells. The results for goblet cell staining with this reagent (AB-PAS) are shown in table :5.6. Representative photographs for both non-infected and infected groups are shown in figure :5.10. The number of goblet cells per duodenal villus in both non-infected and infected vitamin A deficient animals were significantly lower than those of corresponding control and pairfed animals. There was no significant difference in the number of goblet cells per duodenal villus observed between non-infected and infected villi.

Whether rotavirus infection additional to vitamin A deficiency has any effect on goblet cell number or not, vitamin A deficiency alone causes a decrease in the number of goblet cells per villus. Low food intake did not change the number of goblet cells per villus. DeLuca et al (1969) have reported a decrease in the number of goblet cells in the crypt of vitamin A deficient animals. Rojanapo et al (1980) have also shown that the goblet cell number per duodenal crypt of vitamin A deficient animals is about 40% lower than that of controls. The latter group examined only 20 crypts in each of 3 matched deficient and control rats. We present the data for 5 animals in each of the infected groups and 3 in each of the non-infected groups. We measured 20-40 villi in each animal depending upon the availability of longitudinal sections of individual villi, as we measured only those villi which were cut longitudinally from crypt-villus junction to villus tip. Further, Rojanapo et al (1980) have counted crypt goblet cells and we counted villus goblet cells. However, our observation are in general agreement with their results.

Oligomucus cells, the immediate precursor of goblet cells, are located in the lower half of the crypt and the fully differentiated goblet cells eventually migrate towards the villus epithelium, climb to the villus tip and fall into lumen (Merzel and Leblond, 1969). The rate of migration of goblet cells out of the crypt

Table:5.6

Effect of vitamin A deficiency and rotavirus infection on the goblet cell (G.C.) count (Alcian blue-Periodic acid Schiff staining)

Group	Non-infected	Infected
	No.of G.C./Villus	No.of G.C./Villus
Control	12.6±0.2	13.3±0.3
Pairfed	12.7±0.3	12.8±0.3
Deficient	9.9±0.3*	9.8±0.5*

Values are expressed as mean±SEM, 3 sample for Non-infected and 5 sample for Infected group. 20-40 villi in each sample were examined depending upon the availability of the longitudinal villi section.
*Significantly different from corresponding control and pairfed group by "t" test ($P < 0.001$).

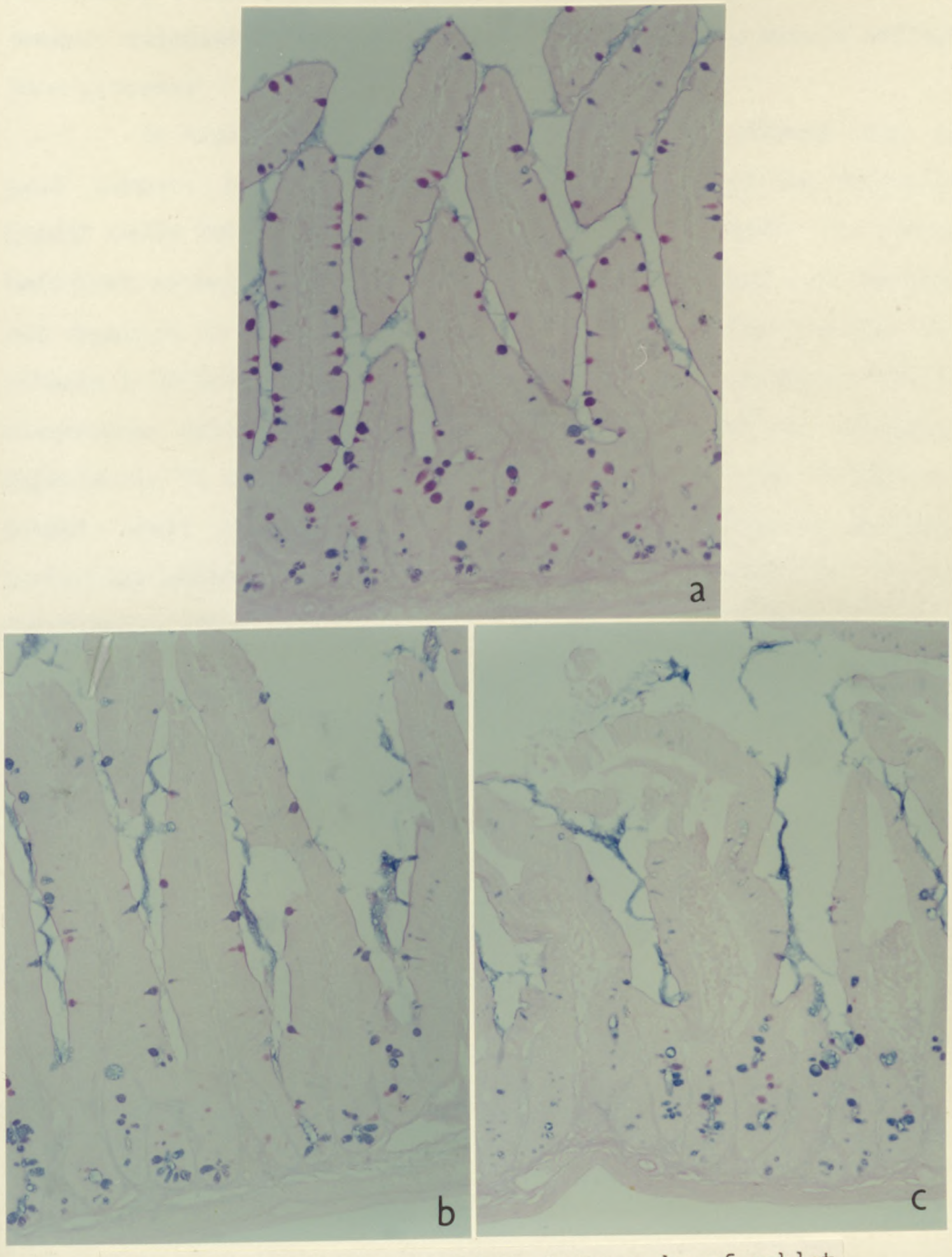


Figure: 5.10. Representative photographs of goblet cell staining in the duodenum of (a) Non-infected pairfed, (b) Non-infected vitamin A deficient and (c) Infected vitamin A deficient mice. AB-PAS stain, X 130.

gland along the villus is known to be unaffected by vitamin A deficiency (Rojanapo et al.,1980). We infer therefore that goblet cell number calculated either per crypt or per villus should reflect the same process.

In explaining how vitamin A deficiency affects the goblet cell number, Rojanapo et al (1980) have shown that the percentage of goblet cells but not oligomucus cells was reduced in vitamin A deficient animals. Further they have shown intestinal cell division is not impaired in vitamin A deficiency. Therefore, they conclude that in vitamin A deficiency, the rate of differentiation of goblet cells from oligomucus cells seems to be reduced. We have not conducted any experiments to determine the mechanism of action of vitamin A on goblet cell number. However, we have investigated whether any particular mucin producing goblet cell is affected by vitamin A deficiency. Thus intestinal sections were treated with either alcian blue to stain the acid mucin producing goblet cells and periodic acid Schiff's reagent to stain the neutral mucin producing goblet cells. The results obtained for acid mucin and neutral mucin staining are shown in table :5.7 and 5.8 respectively. Both types of goblet cells were significantly reduced in number in vitamin A deficient animals compared with control and pairfed animals. The results following rotavirus infection were similar as was the case with the combined PAS-AB staining.

5.3.7. Immunostaining of B and T lymphocytes:

Since the spleen and Peyer's patches of infected vitamin A deficient animals appeared different from those of corresponding control and pairfed groups on histological examination, we decided to measure immuno stained B- and T-cells in the spleen and Peyer's patches of the infected groups. The results for B-cells and T-cells can be expressed in three ways, i) by grading the intensity of colour, ii) by counting the number of stained cells and iii) by measuring the area of B-cells and T-cells stained. The grading of the intensity of

Table:5.7

Effect of vitamin A deficiency and rotavirus infection on the goblet cell (G.C.) count (Alcian blue staining)[1]

Group	Non-infected	Infected
	No.of G.C./Villus	No.of G.C./Villus
Control	11.8±0.3	12.2±0.3
Pairfed	11.5±0.3	11.2±0.3
Deficient	9.8±0.6*	9.2±0.4*

Table: 5.8

Effect of vitamin A deficiency and rotavirus infection on the goblet cell (G.C.) count (Periodic Acid Schiff's staining)[1]

Group	Non-infected	Infected
	No.of G.C./Villus	No.of G.C./Villus
Control	12.2±0.3	13.2±0.3
Pairfed	13.2±0.3	13.1±0.3
Deficient	9.1±0.5*	11.2±0.3*

[1] Values are expressed as mean±SEM, 3 sample for Noninfected and 5 sample for Infected group. 20-40 villi in each sample were examined depending upon the availability of the longitudinal villi section.
*Significantly different from corresponding control and pairfed group by "t" test, (P value at least <0.01).

the colour measurement is not a reliable way to express the results unless there is a great change in colour observed. The intensity of the reaction product may also vary from day to day. We therefore considered counting the number of the B and T cells present. However this was unsatisfactory as the resolution of cell membranes in 8 micron thick frozen sections is unsatisfactory. It is necessary to use frozen sections as the antibodies employed to identify B and T cells do not work in formalin fixed paraffin embedded tissues. Finally, we tried the third approach, that of measuring the areas of B and T-cells. To measure the area we used a computer driven graphics system (model-Apple IIC). All sections were measured under the same magnification (X 10) and same calibration (1000 micron). The area of the field examined was also the same throughout the experiment (4.47 mm^2). The results were expressed as percentage of B-cells and T-cells considering the area of the field examined as 100 percent.

The results obtained for comparative B- and T-cell areas in the spleen sections are shown in table:5.9. Representative photographs for the B and T-cell areas are shown in figure:5.11 and 5.12 respectively. The B-cell areas of vitamin A deficient animals were found to be greater than the pairfed controls, although the difference was not significant. Also, the relative T-cell area of vitamin A deficient animals was found to be lower than that of pairfed control, although the change was not significant. In both cases we used only the pairfed group for comparison as there was apparently no difference between control and pairfed spleens histologically.

During the preparation of the spleen for sectioning, the lymphoid sheaths were cut in different positions. Some were in longitudinal section and some in transverse section. Thus to represent the result as a percentage of B-cells or T-cells may lead to an error in the conclusions. Therefore we expressed our results in terms of B:T cell ratio , which may be more reliable. The results expressed as B:T cell ratio for this experiment are also shown in table :5.9. Although the ratios of B to T cells of infected vitamin A deficient animals were higher than those of pairfed groups, the difference was not

Table: 5.9

Effect of vitamin A deficiency and rotavirus infection on the percentage of B and T-cell area (Mean±SEM)

Group	B-cell area %	T-Cell area %	B/T-cell ratio
Pairfed	33.0±1.2	19.12±3.0	1.92±0.3
Deficient	40.9±4.1	13.85±2.1	3.27±0.6

Difference not significant by "t"test.

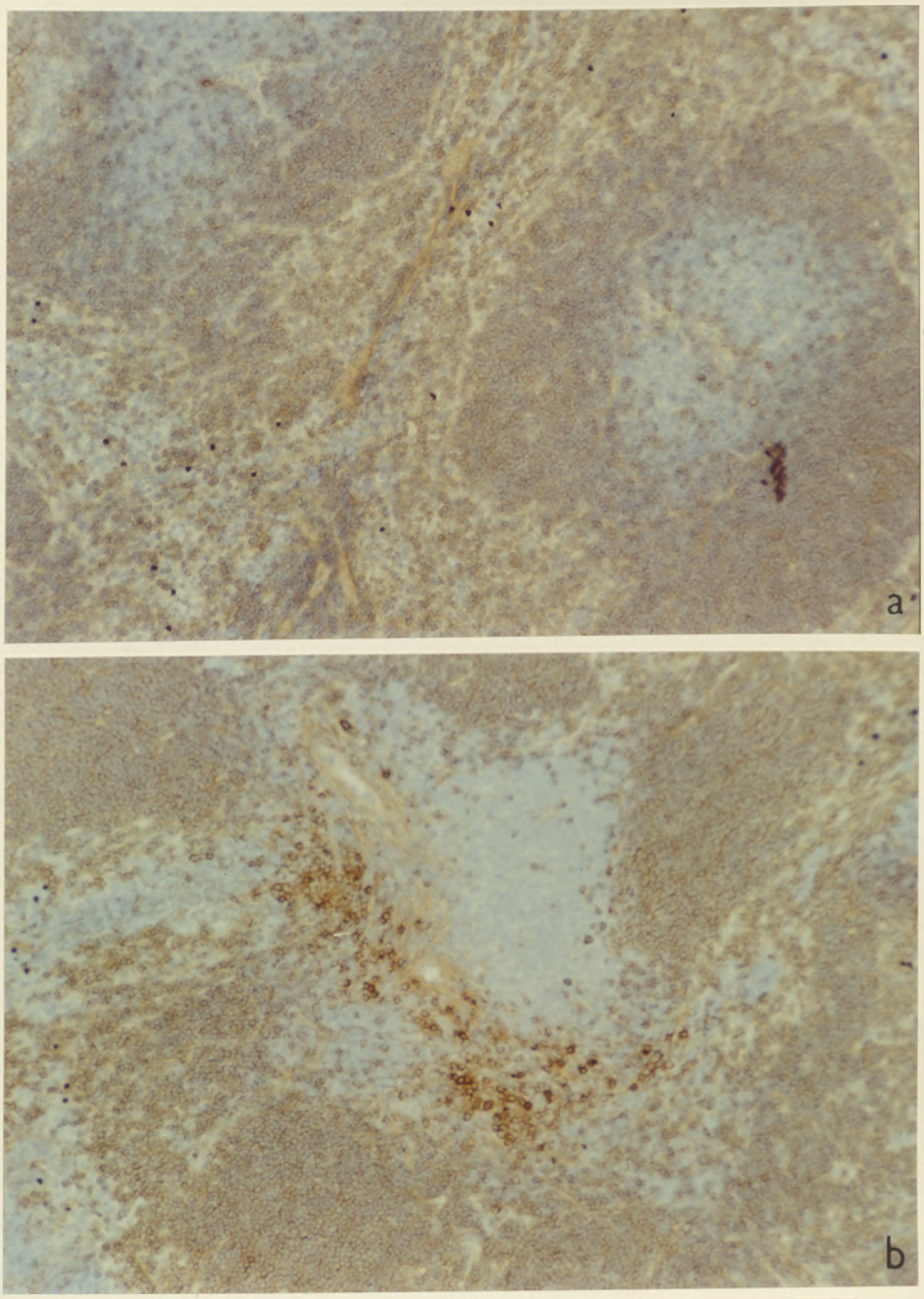


Figure : 5.11. Representative photographs of B cell staining of spleens from (a) infected, pairfed and (b) infected, vitamin A deficient mice. Direct immunoperoxidase staining, X 130.

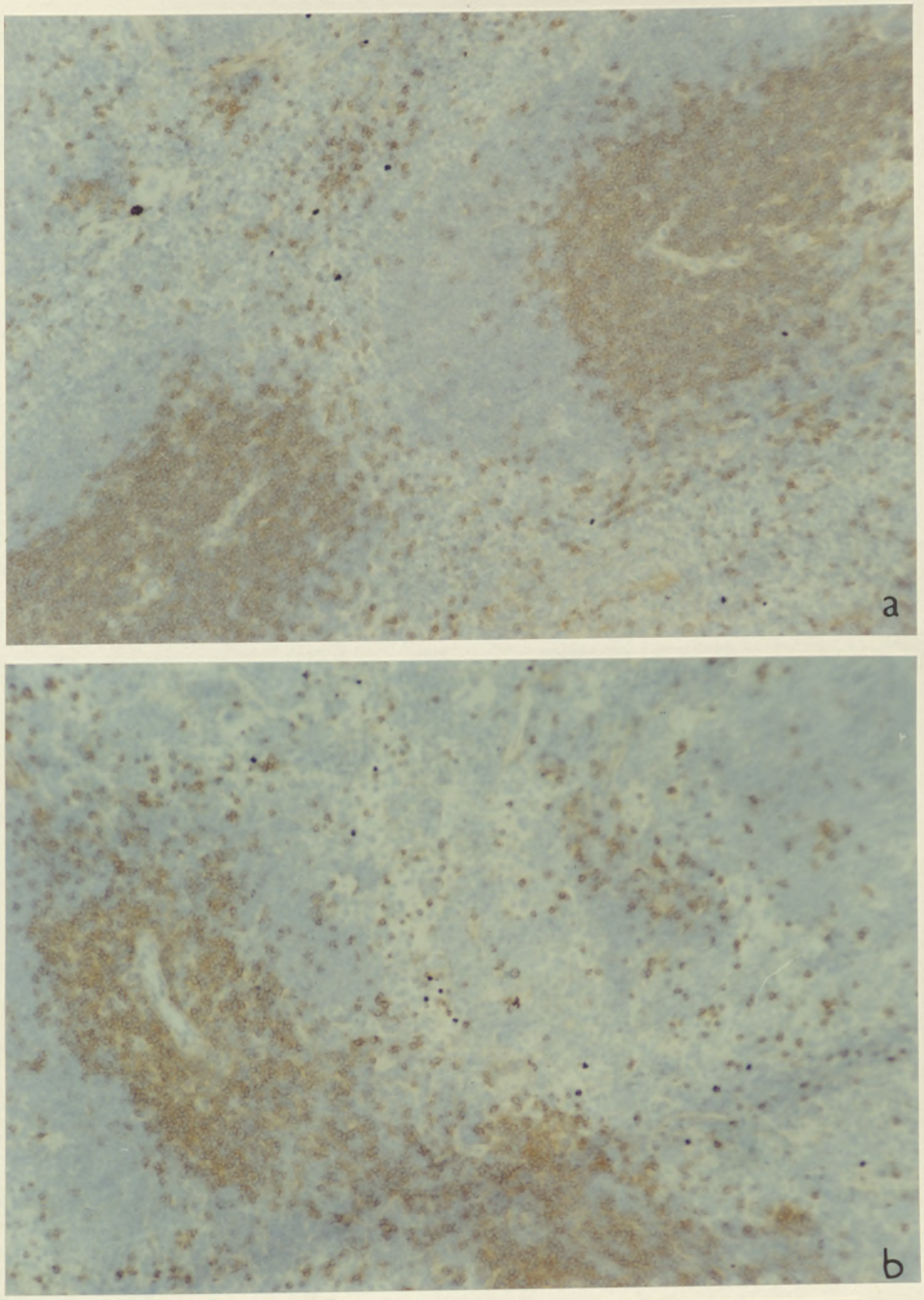


Figure : 5.12. Representative photographs of T cell staining of spleens from (a) infected, pairfed and (b) infected, vitamin A deficient mice. Indirect immunoperoxidase staining, X 130.

significant.

We also stained the Peyer's patches of infected vitamin A deficient and pairfed animals. Since stained cells tend to overlap one another it was not possible to count the numbers of B and T-cells. Further, the measurement of B and T-cell areas was difficult in this tissue.

5.4. Conclusions:

We have investigated the capacity of vitamin A deficient mice to resist rotavirus infection after oral challenge. The results indicate that the intestinal epithelium of vitamin A deficient animals is highly disrupted following rotavirus infection whereas in control and pairfed groups it remains unchanged. It has been reported that rotavirus specifically infects the cells in the upper two thirds of the villus in infant mice (Starkey et al., 1986), and our findings would suggest that vitamin A deficiency increases the susceptibility of these cells to damage by rotavirus. Vitamin A deficiency appears to be insufficient to produce severe damage alone, but does impair the cells' ability to withstand subsequent pathological insult.

Although, a high incidence of infection is known to be associated with vitamin A deficiency in animals and man (Scrimshaw et al., 1968), it is difficult to exclude the effect of low food intake which is associated with vitamin A deficiency. The experimental design in our study included a pairfed group and results of vitamin A deficient animals were compared with the pairfed control rather than the ad libitum control group. Our findings demonstrate the specific effect of vitamin A deficiency and provide convincing evidence on the relationship between rotavirus infection and vitamin A deficiency.

The role of vitamin A in immunity may reside in its protective action at more than one level. The integrity of the epithelial linings and the normal state of mucus secretions are among the factors that may influence invasion by a variety of infectious agents. The mucus barrier of the gastrointestinal tract represents one

component of the non-specific immune system which has been shown to be sensitive to vitamin A status (Rojanapo et al., 1980). The results of the evaluation of non-specific immunity represented by the enumeration of goblet cells in the intestinal villi, indicate that vitamin A deficiency either alone or in presence of infection affects goblet cell differentiation in the villus, whereas low food intake alone does not. As vitamin A deficiency lowers the goblet cells number in intestinal villi, it is possible that it prejudices mucosal immunity non-specifically. This would occur as goblet cells produce mucin, which protects the epithelium of the intestine. Taken together with the marked histological changes seen in the mucosa of the deficient animals infected with rotavirus, we claim an association between reduced number of goblet cells and local damage following virus infection. This indicates an important protective role for intestinal mucin.

The spleen weights of vitamin A deficient infected animals were significantly higher than those of the control and pairfed groups. From all these results we may conclude that as the intestinal mucosal barrier of vitamin A deficient animals is impaired, rotavirus or other agents might have crossed the gut and entered into the general circulation provoking a systemic immune response manifest as an increase in spleen weight.

Although the absolute and relative weight of the thymus was significantly reduced in vitamin A deficient animals both non-infected and infected, we did not see any clear histological changes in the thymus in any of the experimental groups. These findings are at variance with those of Krishnan et al (1974) who observed marked depletion of lymphocytes in the cortex of the thymus in vitamin A deficient animals. However, these workers also reported similar changes in their pairfed animals implying that the changes described can not be attributed to the specific effects of vitamin A deficiency alone, but are non-specific, being more closely related to the degree of general inanition.

In most of the earlier studies in which the interaction of

vitamin A deficiency and immune responsiveness has been explored, the infective challenge has been given by the parenteral route. In the present study we have shown that the non-specific mucosal barrier of the gastro-intestinal tract can be severely impaired by vitamin A deficiency, but that this damage may only become obvious in the face of an infective challenge.

In order to investigate the specific immune response, the total serum antibody levels following oral challenge with rotavirus were measured and the results are presented in the following chapter.

Since the antibody production depends on both B and T cell function, any defect in the antibody production due to vitamin A deficiency , could be due either to defective function of B cells, T cells or both cell types. In order to investigate the T cell function, we further measured the delayed hypersensitivity response of deficient and normal animals.

CHAPTER SIX

EFFECT OF VITAMIN A DEFICIENCY ON THE IMMUNE RESPONSE TO ROTAVIRUS INFECTION

CHAPTER SIX

6.1. Introduction:

In the previous section we have shown that vitamin A deficient mice are more susceptible to rotavirus infection, as revealed by histopathological changes in the gut. We have also shown an impaired non-specific immune response in both the infected and non-infected vitamin A deficient animals, as judged by decreased goblet cell number in the villi of the small intestine. This impaired non-specific immunity could be one of the important reasons for the susceptibility of the gut epithelium to infection. To evaluate specific immunity to rotavirus, it is necessary to investigate antibody levels.

The effect of vitamin A deficiency on antibody production has been pursued extensively. Many of these studies have shown impaired antibody production in the vitamin A deficient subject. For instance, Ludovici and Axelrod (1951) observed that rats fed a vitamin A deficient diet for 4 weeks had lower haemagglutinin titres than control rats when immunized with human RBC by intraperitoneal injection. Pruzansky and Axelrod (1955) reported similar findings following intraperitoneal injection of diphtheria toxoid into rats that had received either a vitamin A deficient diet or a control diet for 12 days. Swine with reduced serum retinol levels (Harmon et al.,1963) and chicks fed a diet with suboptimal vitamin A (Panda and Combs, 1963) showed reduced agglutination responses to Salmonella pullorum bacterial antigen. Vitamin A deficient rabbits (Greene.,1933) and rats (Chandra and Au., 1981) produced low levels of circulating hemolytic antibody when immunized with sheep erythrocytes. Krishnan et al (1974) have shown that vitamin A deficient rats had significantly lower antibody titres against diphtheria and tetanus toxoid as

compared to control and pairfed animals. This group have also shown that there was no significant difference in antibody levels to sheep RBC between pairfed and vitamin A deficient animals. Their results clearly indicate that antibody production to different antigens varies.

Most of these investigations showed impaired antibody production in the vitamin A deficient animals when antigen was injected by either the intraperitoneal or by the intramuscular route. Very few studies have been carried out to investigate the effect of vitamin A deficiency on the antibody production following oral feeding of antigen (Sirisinha et al.,1980).

Therefore, the present study was designed to investigate the effect of vitamin A deficiency on antibody production following oral feeding of live rotavirus. In addition to antibody levels we also investigated delayed hypersensitivity, as a measure of T-cell function.

6.2. Experimental procedure:

The study protocol is presented in figure :6.1. The routine procedures are already described in chapter-2.

6.2.1. Procedure for immunization:

Male Porton mice, 19-21 days old, were initially weight matched and divided into four groups, as control, pairfed and two vitamin A deficient groups. They were supplied with their respective diet as described in chapter-2.2. One vitamin A deficient group was refed vitamin A diet (control diet) after 10 weeks of feeding experimental diet and termed "vitamin A refed". The weights of the animals were taken weekly and food consumption measured daily. All animals were kept on their respective diet for 11 weeks (77 days) and then each group was divided into two subgroups as described previously (chapter-5).

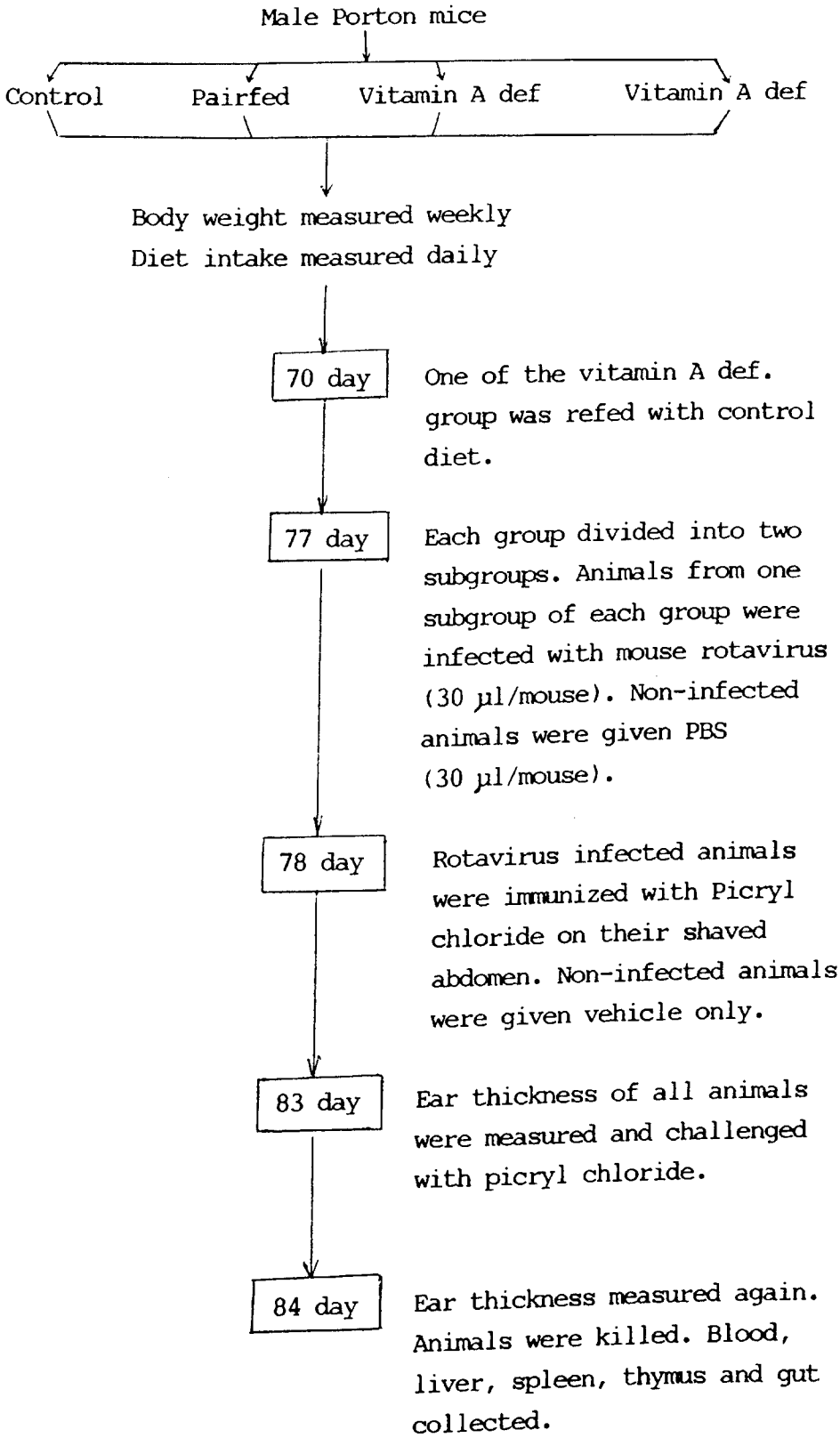


Figure: 6.1 Scheme of the procedure for the study of the immune response in the vitamin A deficient mice.

6.2.1.1. Antibody production:

On day 77, animals from one subgroup were infected with 30 µl/mouse of EDIM rotavirus (containing $10^{4.5}ID_{50}$)by oral dosing and termed the "infected group". Animals from the other subgroup were dosed with 30 µl/mouse of phosphate buffer saline (PBS) and treated as non-infected. 7 days post-dosing blood samples were collected.

6.2.1.2. Delayed hypersensitivity test:

For the demonstration of delayed hypersensitivity, on day 78 (one day after rotavirus dosing) the animals in the infected group were immunized with 150 µl and 50 µl of 5 % Picryl chloride in acetone:alcohol (1:9 vol) on the shaved abdomen and to the 4 foot pads. Non-infected animals received the same amount of acetone and alcohol solution on the shaved abdomen and to the 4 foot pads. 24 hours before killing the ear thickness of all animals was measured by micrometer followed by challenged with 1 % picryl chloride solution in acetone: olive oil (1:9 vol) on both ears. On day 84 or one week post-dosing, the ear swelling of all animals was measured.

The animals were bled by heart puncture and blood samples were allowed to stand at room temperature for one hour to allow clot formation. After clotting, the blood was centrifuged and the clear serum removed with a pasteur pipette. The serum samples were stored in ependorff tubes at -20°C until analysed. Immediately after the blood was collected, the carcase of the animals was cut open and the liver, spleen, and thymus were excised and weighed. Liver was then stored at -20°C until analysed for vitamin A content. Spleen, thymus, a representative portion of small intestine and duodenum were fixed in 10 % buffered formalin solution for histological analysis.

6.2.2. Determination of vitamin A:

6.2.2.1. Liver:

Liver vitamin A was determined by the method of Bayfield (1975) with the modifications (Section-2.3).

6.2.2.2. Serum:

Serum vitamin A was estimated by the high performance liquid chromatography (HPLC) method of Bieri et al (1979) with slight modification (Section-2.4.2).

6.2.3. Histological study:

The histology of the gut (middle portion of the small intestine) was examined by the method described in section-2.5.

6.2.4. Antibody assay:

Total circulating antibody specific to rotavirus antigen was measured by using the ELISA method described in section-2.8.2.

6.2.5. Cell mediated immunity:

Delayed type hypersensitivity was measured by using the method described in section-2.9.

6.2.6. Statistical analysis:

Results are expressed as the mean and standard error of the mean. Differences between groups were evaluated by using 2-way analysis of variance (ANOVA) for independent means for the parametric data and Wilcoxon rank sum test for the non-parametric data.

Differences of the means were considered significant when $P<0.05$.

6.3. Results and discussions:

6.3.1. Body weights:

The gain in body weight of all groups of non-infected and infected animals throughout the experiment is shown in figure-6.2. The body weight of non-infected vitamin A deficient animals reached a plateau at about 10 weeks on the experimental diet and thereafter remained steady. Both the control and pairfed animals gained weight till the end of the study. An additional group of non-infected vitamin A deficient animals, refed vitamin A diet (control diet) after 10 weeks on the deficient diet showed a further increase in the body weight.

After 11 weeks on the experimental diet, vitamin A deficient animals had a significantly lower body weight compared with control and pairfed animals irrespective of infection ($P<0.01$, univariate level). The body weight of the vitamin A refed animals was also significantly lower than those of control and pairfed group irrespective of infection ($P<0.01$, univariate level). Although the vitamin A refed group showed an increase in body weight, there was no significant difference in body weight between vitamin A deficient and vitamin A refed animals irrespective of infection. Pairfed and control animals did not differ significantly.

At 12 weeks, the body weight of the control and pairfed group was significantly higher than the vitamin A deficient group ($P<0.01$) and vitamin A refed group ($P<0.05$) irrespective of infection (univariate level). There was no significant difference in body weight observed between either the control and pairfed groups or vitamin A deficient and vitamin A refed groups. Also there was no significant difference observed between infected and non-infected mice irrespective of dietary treatment. Although the infected animals of all groups showed a fall in the body weight after the rotavirus and picryl chloride immunization. We did not see any reduction in body

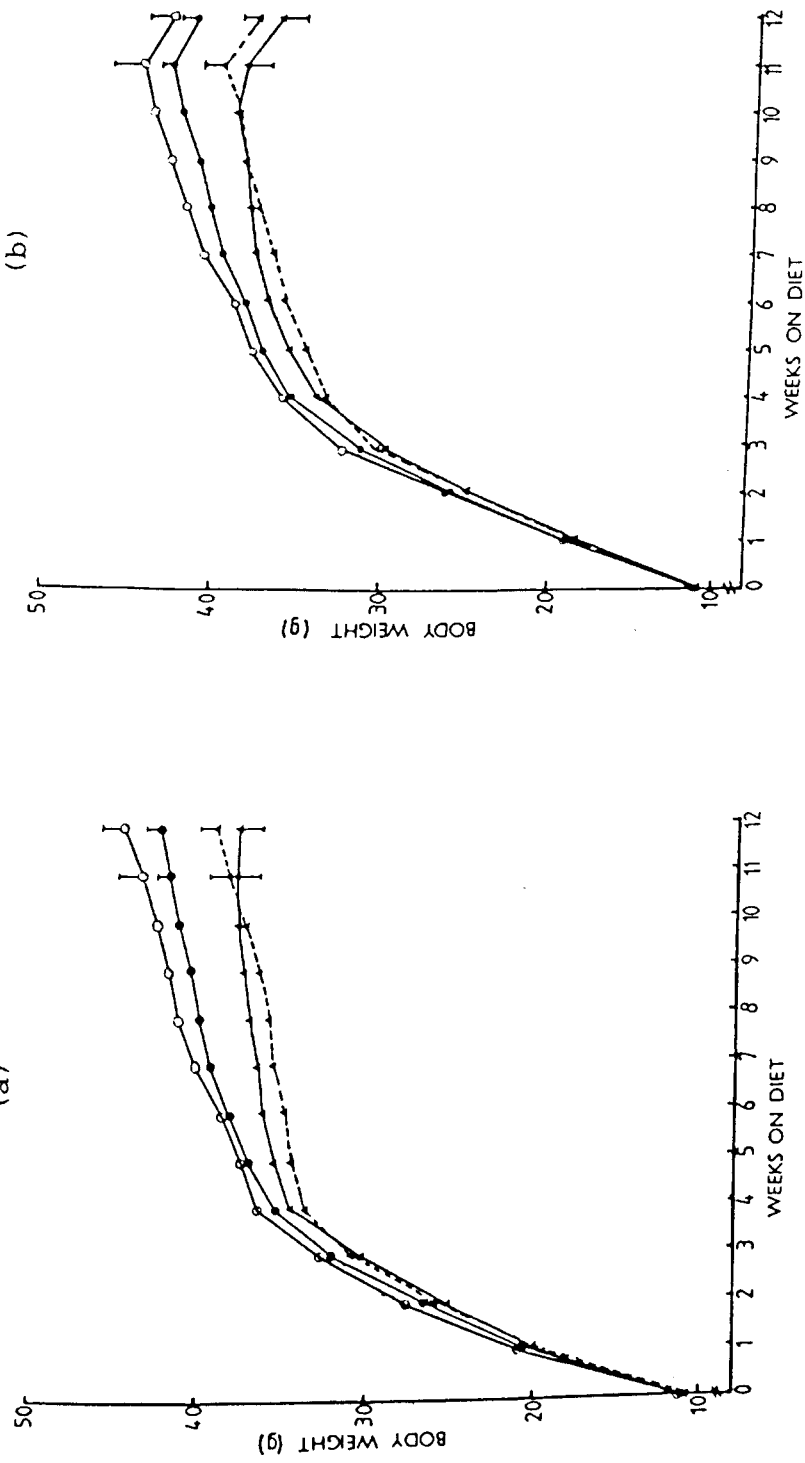


Figure: 6.2 Body weight gain of different groups of (a). non-infected and (b) infected animals during the experiment. Control (●—●), pairfed (●—■), vitamin A deficient (▲—▲) and vitamin A refed (▲---▲). Each point shows the mean of 5 animals. The 11 and 12 weeks time point display the mean+SEM.

weight after infection with rotavirus alone. The body weight loss in the present study could either be due to picryl chloride challenge alone or to a synergistic effect of picryl chloride and rotavirus.

6.3.2. Diet intake:

The daily diet intakes of different groups of non-infected and infected animals are shown in figure: 6.3 and 6.4 respectively. In the non-infected group, the intake of vitamin A deficient animals was slightly lower than the control animals after 9-10 weeks of feeding. On the other hand, when the vitamin A deficient animals were refed a vitamin A containing diet after 10 weeks, they increased their diet intake compared with the vitamin A deficient group.

The diet intake pattern of infected animals was similar to non-infected animals up to 11 weeks of feeding. After infection and immunization at 11 weeks, animals from all infected groups showed a sharp fall in food intake for the first few days and thereafter the intake was returned to normal (figure: 6.5). When we infected the animals with rotavirus only, we did not see any reduction in food intake in any of the groups.

6.3.3. Organ weights:

6.3.3.1. Liver weight:

The liver weights of the non-infected and infected groups are shown in table: 6.1. Total liver weights of vitamin A deficient animals were significantly lower than those of control ($P<0.01$), pairfed ($P<0.01$) and vitamin A refed animals ($P<0.05$) irrespective of infection (univariate level). There was no significant difference in liver weight between any other dietary groups.There was no significant difference observed between infected and non-infected groups. Further rotavirus infection did not significantly influence liver weight. The data suggest that the liver weight increased towards normal when

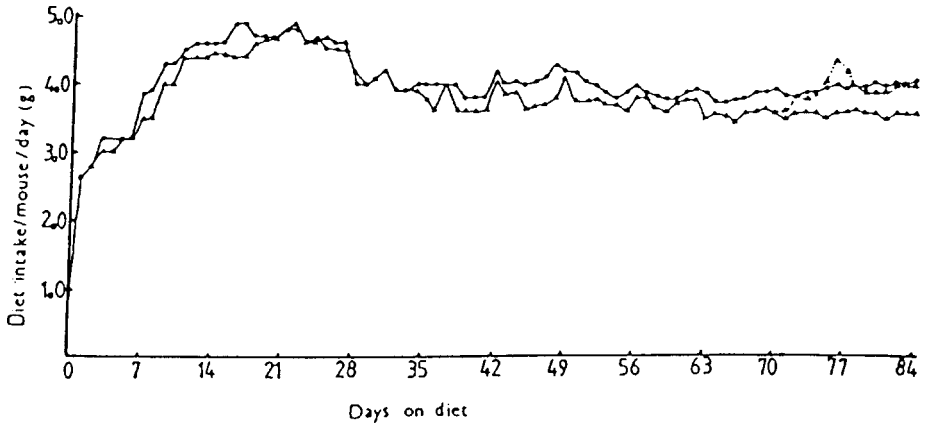


Figure: 6.3 Daily diet intake of the non-infected control ad libitum (●——●), vitamin A deficient (▲——▲) and vitamin A refed (△---△) animals throughout the study.

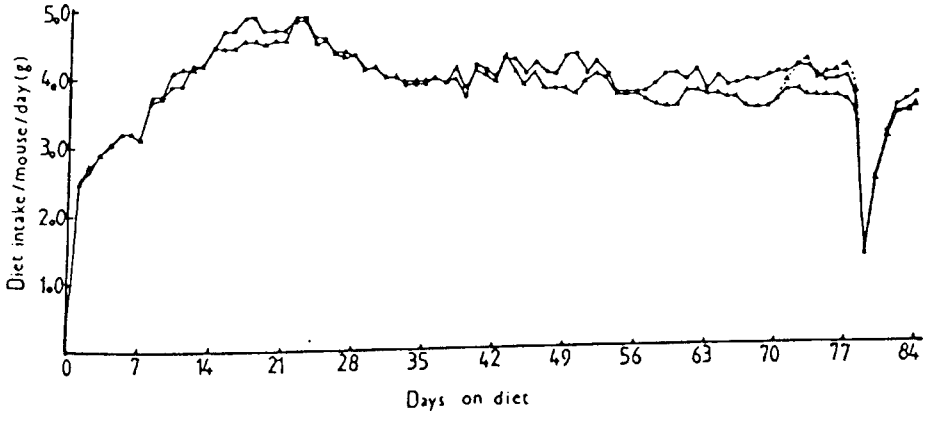


Figure: 6.4 Daily diet intake of the infected control (●——●), vitamin A deficient (▲——▲) and vitamin A refed (△---△) animals throughout the study.

Table: 6.1

Effect of vitamin A deficiency and rotavirus infection on the liver weight[1]

Group	Total weight		As % of Body weight	
	Infected g	Non-infected g	Infected"" %	Non-infected %
Control	1.90±0.06	1.93±0.08	4.45±0.05	4.31±0.14
Pairfed	1.81±0.04	1.84±0.04	4.39±0.11	4.34±0.05
Deficient"	1.66±0.04	1.47±0.08	4.58±0.18	3.90±0.22
Vit.A refed	1.76±0.05	1.77±0.08	4.65±0.12	4.54±0.27

[1]Results are expressed as the Mean±SEM of 5 samples in each group.
"Significantly different from control and pairfed group ($P<0.01$), and refed vitamin A group ($p<0.05$) irrespective of infection, when compared for the total weight.
""Significantly different from the non-infected animals ($P<0.05$) irrespective of any dietary treatment, when compared for the relative weight.

vitamin A deficient animals were refed a vitamin A diet (control diet). Therefore the effect of vitamin A deficiency on the total liver weight is reversible. Finally, infection does not have any effect on liver weight over and above that due to vitamin A deficiency.

There was no significant change in the relative liver weight (liver weight as % of body weight) between any of the dietary groups irrespective of infection. This result is consistant with our previous findings. The relative liver weights of the infected animals were significantly higher than those of non-infected group irrespective of any dietary treatment ($P<0.05$, univariate level). This is not in agreement with our previous results. A possible explanation is that in the present study two antigens (rotavirus and picryl chloride) were used. However, as the body weights decreased in all groups of infected animals this would result in an increased relative liver weight. Since total liver weight was not altered by infection, it would appear that infection of itself does not affect liver mass.

6.3.3.2. Spleen weight:

The spleen weights of both the non-infected and infected animals are shown in table: 6.2. Total spleen weights of the infected animals were significantly higher than those of the non-infected animals irrespective of dietary treatment ($P<0.05$, univariate level). The diet alone had no effect on the total spleen weight but in the presence of infection there was a significant increase ($P<0.05$). In other words, diet influences the total spleen weight in response to infection. We observed that following infection, the vitamin A deficient spleens were significantly heavier than either of the non-infected groups or the infected, vitamin A restored animals. There was no significant difference in spleen weight between infected deficient animals and the infected control and pairfed groups, athough the absolute mean of the spleen weight of vitamin A deficient animals was higher. The total spleen weights of the vitmin A refed animals were similar to the control and pairfed groups.

Table: 6.2

Effect of vitamin A deficiency and rotavirus infection on the spleen weight[1]

	Total weight		As % of body weight	
Group	Infected* mg	Non-infected mg	Infected* %	Non-infected %
Control	118.1±7.2	94.4±3.0	0.29±0.03	0.21±0.01
Pairfed	113.2±8.3	104.2±5.7	0.27±0.02	0.25±0.01
Deficient+	157.4±20.9**	96.2±6.7	0.44±0.08	0.26±0.02
Vit.A refed	103.9±10.9	100.3±9.2	0.27±0.05	0.26±0.02

[1]Results are expressed as the Mean±SEM of 5 samples in each group.

*Significantly different from the corresponding non-infected group irrespective of dietary treatment ($P<0.05$).

**Significantly different from all non-infected group and infected refed group ($P<0.05$), when compared for the total weight.

+Significantly different from control ($P<0.05$), Pairfed and refed group ($P=0.05$) irrespective of infection, when compared for the relative weight.

The relative spleen weights (% of body weight) of the infected animals were significaltly higher than the non-infected group irrespective of any dietary treatment ($P<0.01$, univariate level). The relative spleen weights of infected vitamin A deficient animals appeared higher than any other infected group. However, when we performed two way analysis of variance, the relative spleen weight of the vitamin A deficient animals was significantly higher than control ($P<0.05$) and pairfed animals ($P=0.05$) irrespective of infection. There was no significant difference in either total or relative spleen weight in the vitamin A refed group when compared to control and pairfed animals. No significant interaction was observed between diet and infection. Relative spleen weights of control and pairfed groups were similar.

6.3.3.3. Thymus weight:

The thymus weights of both the infected and non-infected groups are shown in table: 6.3. Total thymus weights of the infected group were significantly lower than those of the non-infected group irrespective of any dietary treatment ($P<0.01$, univariate level). There was a significant interaction observed between diet and infection ($P<0.05$). We also observed that the total thymus weight of the non-infected vitamin A deficient animals was significantly lower than the control and pairfed groups ($P<0.05$) but not the vitamin A refed group. There was no significant difference in total thymus weight between control, pairfed and vitamin A refed groups.

These results suggest that total thymus weight is affected by the vitamin A deficiency alone or in combination with low food intake. But low food intake alone does not have any effect on thymus weight. This result is in agreement with our previous findings. The data also show that following refeeding with vitamin A deficient animals rapidly recover their thymus weight. The reduced thymus weight in all the infected animals in this study is difficult to explain. In our previous study, infection with rotavirus caused further weight loss

Table: 6.3

Effect of vitamin A deficiency and rotavirus infection on the thymus weight[1]

	Total weight		As % of body weight	
Group	Infected* mg	Non-infected mg	Infected* %	Non-infected %
Control	19.7±0.5	51.3±1.6	0.046±0.002	0.115±0.004
Pairfed	21.2±1.0	50.4±2.2	0.051±0.003	0.119±0.007
Deficient	20.7±2.7	41.0±3.4	0.057±0.007	0.109±0.01
Vit.A refed	19.3±1.0	49.2±2.1	0.051±0.003	0.126±0.003

[1]Results are expressed as the the Mean±SEM of 5 samples in each group.
*Significantly different from the corresponding non-infected animals ($P<0.01$) irrespective of any dietary treatment.

only in the vitamin A deficient group. The weight loss of the thymus in the present study could be either due to picryl chloride alone or because of the cumulative effect of both picryl chloride and rotavirus.

The relative thymus weights (% of body weight) of the infected animals was significantly lower than those of the non-infected group irrespective of dietary treatment ($P<0.01$, univariate level). Diet alone had no effect on the relative thymus weight. There was no significant interaction observed between diet and infection.

6.3.4. Vitamin A levels:

6.3.4.1. Liver vitamin A:

Liver vitamin A content of both the non-infected and infected animals are shown in table: 6.4. The total liver vitamin A content of the vitamin A deficient group was significantly lower than control, pairfed ($P<0.01$) and the vitamin A refed group ($P<0.05$) irrespective of infection (univariate level). There was no significant difference observed between control and pairfed animals. The vitamin A refed group regained their vitamin A stores but at a significantly lower level than control and pairfed animals ($P<0.01$). There was no significant difference observed between infected and non-infected groups. Further there was no significant influence of rotavirus infection on the liver vitamin A content in combination with diet.

6.3.4.2. Serum vitamin A:

Serum vitamin A levels for both infected and non-infected animals are shown in table: 6.5. The serum vitamin A levels of the vitamin A deficient group were significantly lower than any other dietary group irrespective of infection ($P<0.01$, univariate level). Vitamin A levels in the serum of refed animals was also significantly

Table: 6.4

Effect of vitamin A deficiency and rotavirus infection on the liver vitamin A[1]

Group	Infected µg/liver	Non-infected µg/liver
Control	232.4±8.7	235.8±8.5
Pairfed	215.8±9.8	227.4±6.8
Deficient""	0.5±0.1	0.4±0.1
Vit.A refed""	13.3±0.5	15.6±0.7

Table: 6.5

Effect of vitamin A deficiency and rotavirus infection on the serum vitamin A[1]

Group	Non-infected (5) µg/100ml	Infected" (4) µg/100ml
Control	47.2±1.3	19.1±1.1
Pairfed	43.6±4.3	17.7±1.1
Deficient""	5.1±0.4	2.0±0.1
Vit.A refed""	34.6±3.6	9.9±0.5

[1]Results are expressed as the Mean±SEM od 5 samples in each group.

"Significantly different from non-infected animals ($P<0.01$) irrespective of any dietary treatment.

""Significantly different from any other group ($P<0.01$) irrespective of infection.

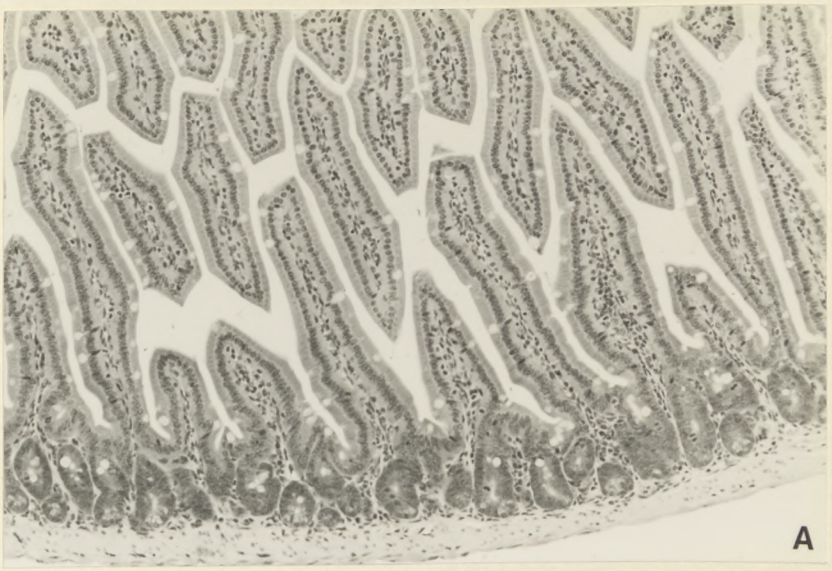
lower than control and pairfed animals irrespective of infection ($P<0.01$). There was no significant difference observed between control and pairfed animals. The circulating vitamin A levels of infected animals were significantly ($P<0.01$) lower than those of non-infected animals irrespective of any dietary treatment. We also observed significant ($P<0.01$) interaction between diet and infection. Since previously we did not measure serum vitamin A from the rotavirus infected animals, it is at present difficult to attribute the reduced serum vitamin A levels to the rotavirus infection, to picryl chloride or to mixed antigen challenge.

6.3.5. Histology of the gut:

The histology of both the non-infected and infected gut (middle portion of the small intestine) was examined under the light microscope. The histology of the non-infected gut appeared normal and had intact villi, consistant with our previous findings.

Significant changes in the infected vitamin A deficient gut were observed when compared to infected, control and pairfed groups. About 80 % of the infected vitamin A deficient animals had extensive histopathological changes. On the other hand, infected control and pairfed animals showed a normal gut histology. The tips of villi from the infected vitamin A deficient animals were almost destroyed and some of them appeared foamy and vacuolated whereas in the infected control and pairfed animals the villus tips were intact. The vitamin A deficient group, which was refed vitamin A diet (control diet), showed a comparatively more normal gut histology as compared to the infected vitamin A deficient group. About 60 % of the animals in vitamin A refed group showed intact villus tips. The rest of the animals in this group had villus destruction resembling the infected vitamin A deficient group. Representative photographs for the gut histology are shown in figure: 6.5. The gut histology of the infected vitamin A deficient animals in this study is consistant with the finding of our

Figure: 6.5. Representative photographs of the histology of the infected gut (small intestine) of (A) Pairfed (B) vitamin A deficient and (C) vitamin A refed mice. H & E stain, X 112 for (A), X 90 for (B) and (C).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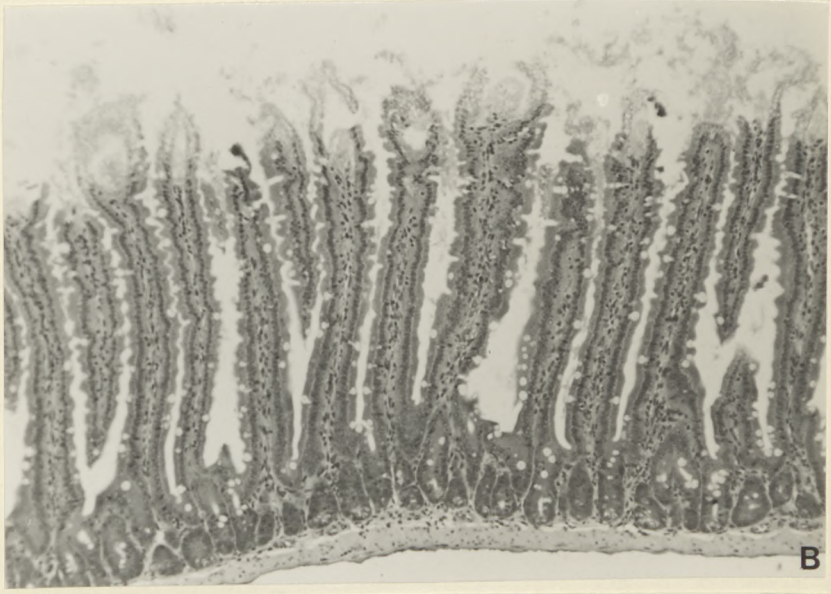
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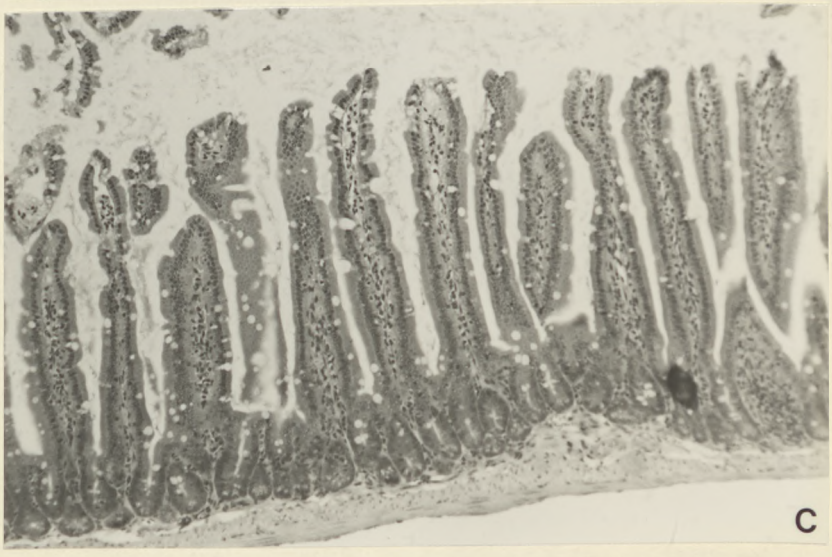
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previous experiment, this confirms our suggestions that rotavirus infection in the presence of vitamin A deficiency caused histopathological changes in the gut. Refeeding with control diet one week before infection gave partial protection to the gut. This finding indicates that refeeding with vitamin A can reverse the effect of vitamin A deficiency on the gut.

6.3.6. Serum antibody levels:

Initially the levels of serum antibody directed towards rotavirus antigen were investigated using a cell-ELISA method (Section:2.8.1). In general the results obtained by this technique were not satisfactory. This method requires the growth of monolayers of monkey kidney epithelial cells in 96 well culture plates with the subsequent infection of cells growing in each well with rotavirus. Although the same amount of virus suspension is used to infect the monolayer in each well of the plate, small variations in the growth of the monkey kidney cells lead to variability in the antibody titres observed in replicate wells within the assay. Microscopic examination of the monolayers growing in 96 well plates following infection revealed some variation and detachment of infected monkey kidney cells. Hence the results which follow (figure: 6.6) were obtained using the cell lysate ELISA technique described in the materials and methods (section: 2.8.2).

The total serum antibody levels, specific to rotavirus, of each of the dietary groups are shown in figure: 6.6. The data showing the units of rotavirus antibody were calculated from the standard curve obtained with the standard rotavirus serum (Mouse anti-rotavirus) in each ELISA plate. The units of anti-rotavirus activity were defined for the standard anti-rotavirus serum as the dilution of serum X($1x10^6$) (See chapter-2.8.2). In each group the results include the data from the previous experiment in order to expand the sample size. Although the two studies were carried out at different times, we performed these studies under the same

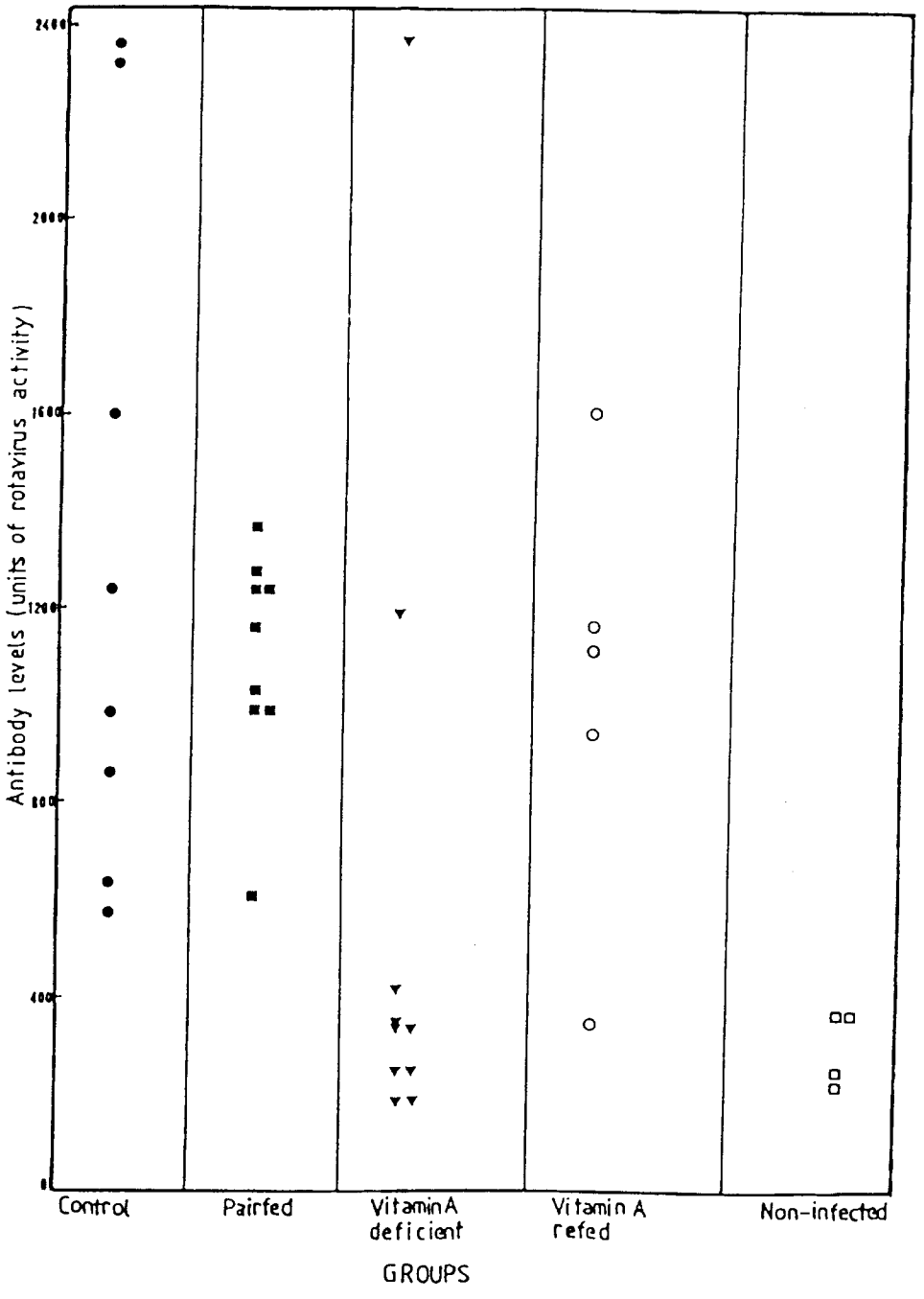

Figure: 6.6 The distribution of antibody levels specific for rotavirus in different dietary groups following oral challenge. For infected animals, each point represents an individual antibody level. In non-infected controls the mean antibody levels are given for each dietary group.

experimental conditions and this permits us to combine the data for statistical analysis.

The mean antibody levels of different groups are shown in table: 6.6. The antibody levels of vitamin A deficient animals were significantly lower than those of the control and pairfed groups ($P<0.05$). Although the vitamin A refed group had higher mean antibody levels than the vitamin A deficient group the difference did not reach statistical significance. Furthermore, the mean antibody levels of vitamin A refed group appeared lower than either the control or pairfed animals, this difference was also not statistically significant. We measured rotavirus antibody levels from the non-infected animals, where the antibody units displayed show the mean of all groups. The serum samples from each group were pooled and the antibody levels measured. The antibody levels of the non-infected animals served as the background anti rotavirus titre in these animals. Since these mice were conventional animals they had previously been exposed to rotavirus and had low levels of rotavirus antibody in the circulation.

As we infected rotavirus by the oral route, one would expect an increase in secretory IgA directed to rotavirus antigens in the gut and possibly an increase in IgA levels in the circulation. Therefore it is appropriate to measure the IgA levels either in secretion or in serum. We intended to investigate the serum IgA levels, however we failed to measure the IgA. The main reason was the unavailability of pure mouse anti rotavirus IgA. Secondly most of the commercially available anti-mouse IgA antibodies cross reacted with other immunoglobulins. Hence we decided to measure the total serum antibody specific to rotavirus. In this study we also tried to investigate the IgG levels specific to rotavirus, again we failed to demonstrate any change of IgG levels. The IgG levels in these animals were very low and were similar to non-infected animals. This could reflect the route of immunization, where mainly IgA responses were expected.

Krishnan et al (1974) have shown significantly reduced antibody levels in vitamin A deficient rats given tetanus and

Table: 6.6

Effect of vitamin A deficiency on the total serum antibody levels specific to rotavirus(Mean±SEM)[1]

Group	Sample no (n)	antibody levels Units
control	8	1299.0±236.0
Pairfed	9	1100.0±76.0
Vitamin A deficient	10	594.0±217.0 *
Vitamin A refed	5	1031.0±202.0

[1] Results are expressed as the units of anti rotavirus activity extrapolated from the standard curve of the standard serum (See chapter-2.8.2).

* Values significantly ($P<0.05$) different from the control and pairfed animals by Wilcoxon rank Sum test.

diphtheria toxoid compared with those of control and pairfed animals. These authors have also shown lower antibody levels in the pairfed group when compared with the control group, although only 3 male Holtzman albino rats were present in each group. The present study failed to show a significant difference in antibody levels between pairfed and control animals, in disagreement with Krishnan et al (1974). The reasons for this could be many, for instance, the route of immunization and type of antigen used. Krishnan et al (1974) have further shown that there was no significant difference in antibody levels against sheep RBC between pairfed and Vitamin A deficient animals, this finding clearly indicates that antigens vary in their immunological behavior. Moreover these authors immunized intramuscularly to stimulate systemic immunity, whereas we used the oral route for immunization.

Smith and Hayes (1987) have investigated the IgM, IgG1 and IgG3 responses to hemocyanin and lysozyme given intraperitoneally. These authors have shown no significant change in the IgM levels between vitamin A deficient and control mice in the early stage of vitamin A deficiency (serum retinol concentration was 46 % of controls). However the IgG1 response was <30% and IgG3 response <20% of control response. As the deficiency progressed (serum retinol 20 % of control) the vitamin A deficient mice produced only 70 % of the IgM levels of control mice. The IgG1 response was still <30% and IgG3 response was <3% of the control values. This group also demonstrated that the immune response to lysozyme was weaker than that to hemocyanin. This study indicates that the effect of vitamin A deficiency on antibody production depends on the severity (stage) of the deficiency, type of antigen and the isotype of the immunoglobulin response measured. Sirisinha et al (1980) have shown that local anti-DNP responses in vitamin A deficient rats were depressed compared with control animals. On the other hand, the systemic anti-DNP responses (serum antibody) were only affected marginally by vitamin A deficiency. These authors immunized the same rats by oral feeding and intraperitoneal injection of the DNP-BCG at the same time, their

immunization protocol is therefore quite different from our study.

Harmon et al (1963) have also shown that the net serum antibody titres of vitamin A deficient pigs were significantly lower than those of control pigs following immunization with phenolized Salmonella pullorum antigen intraperitoneally. This group have also shown that after a 6 to 7 week repletion period the formerly vitamin A deficient pigs had a level of haemagglutination response to human erythrocytes, which was similar to control pigs which had received the complete diet throughout the study. Our study has shown improved antibody titre in the vitamin A refed group as compared to vitamin A deficient animals, although statistically the difference was insignificant. Since there was no significant difference observed between the vitamin A refed group and either control or pairfed animals, the present study implies a better antibody response against rotavirus infection after vitamin A refeeding one week prior to infection. The results of Harmon et al (1963) differ from our result in that the differences in antibody production in the repleted animals may be due to the difference in the period of refeeding of control diet to the formerly vitamin A deficient animals.

6.3.7. Cell mediated immunity:

We have seen from chapters-3 &5 that thymus weight is affected by the vitamin A deficiency , we wanted to investigate the effect of vitamin A deficiency on T cell function. Since delayed type hypersensitivity (DTH) responses are a correlate of cell mediated immunity (Roitt et al., 1985), we decided to investigate the DTH responses to the skin contact antigen, trichloro-nitro-benzene (Picryl chloride). The delayed-type of hypersensitivity responses (as expressed by the percentage in increase in ear thickness) of all dietary groups are shown in figure:6.7. The DTH responses of the picryl chloride immunized animals were significantly higher than the non-immunized animals irrespective of any dietary treatment. The DTH responses of the vitamin A deficient mice were significantly lower

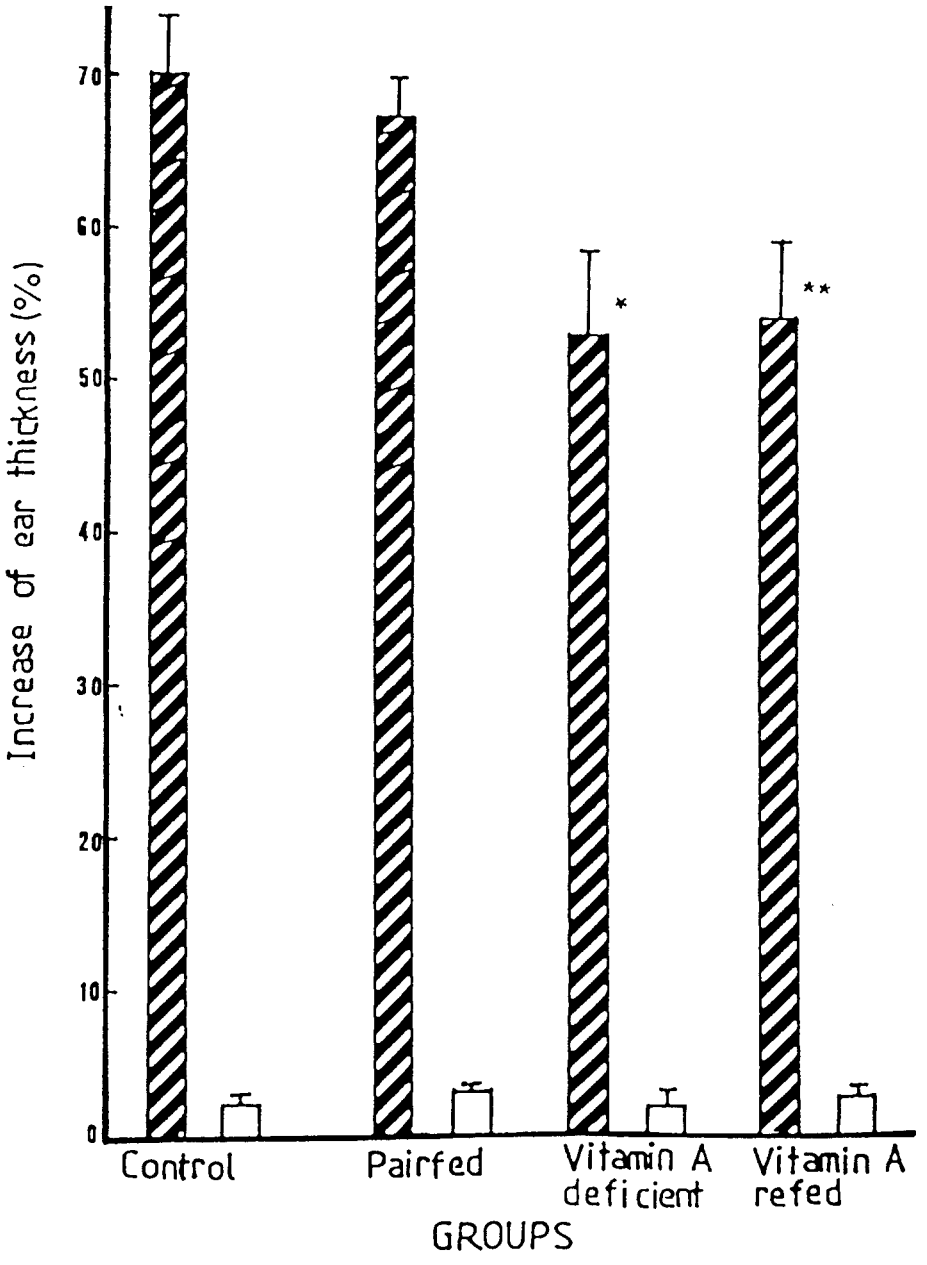

Figure: 6.7 The delayed type hypersensitivity responses of different dietary groups to the skin contact allergen picryl chloride. Infected (▨) and non-infected (□). *Significantly different from the control and pairfed group ($P<0.05$). **Significantly different from control group ($P<0.05$).

than control animals irrespective of infection. There was no significant difference in DTH response between vitamin A deficient and pairfed animals irrespective of infection. Further we observed a significant ($P<0.05$) interaction between diet and immunization. Therefore, when the combined effect of diet and immunization is considered, vitamin A deficient animals had significantly lower DTH responses compared with control and pairfed animals ($P<0.05$). The DTH response of the vitamin A refed group was significantly reduced when compared with the control group ($P<0.05$). However there was no significant change observed between either the pairfed and vitamin A refed group or vitamin A deficient and vitamin A refed group. We refed the control diet to the previously vitamin A deficient mice for only two weeks and these animals failed to show improved DTH responses. This study indicates that vitamin A deficiency leads to functionally defective cell mediated immunity. It is possible that if the refeeding time had been increased the DTH response would have returned to normal levels.

Smith et al (1987) have shown that vitamin A deficient mice at either an early or a late stage of deficiency, have a significantly reduced DTH responses when compared with control animals. This group used dinitroflurobenzene as a skin contact antigen, and their results are similar to our findings.

The delayed type hypersensitivity response involves antigen uptake and processing by macrophages followed by T-cell sensitization, lymphokine release and migration of macrophages to the site of antigen challenge (Roitt et al., 1985). Smith et al (1987) have suggested that one or several of these steps may be decreased in vitamin A deficient mice. However they were unable to explain the diminished DTH response in the vitamin A deficient mice on the basis of T cell number and distribution, since these remained unaltered. Similarly when we examined the T cell areas in spleen sections by using a T cell marker we were not able to identify any significant changes between pairfed and vitamin A deficient animals.

The effect of vitamin A deficiency on the cell mediated

immune function has been examined in vitro by many workers. For instance, Nauss et al (1979) have shown that splenic lymphocytes from deficient rats had one third of the transformation response to the mitogens Concanavalin A (Con A), Phytohemagglutinin (PHA) and E.Coli lipopolysaccharide S (LPS) compared to control and pairfed animals. This group did not see any significant difference in thymus lymphocyte transformation in response to Con A, as a result of vitamin A deficiency. Further, when the vitamin A deficient rats were supplemented with Vitamin A, the transformation response returned to control values within 3 days. These studies all show that the vitamin A deficiency impairs cell mediated immunity either in vivo or in vitro.

6.4. Conclusions:

The present study was designed to investigate the relationship between vitamin A deficiency and the immune response to rotavirus infection, in an attempt to explain the pathological changes seen in the gut of vitamin A deficient animals after rotavirus infection.

The study presented here shows that after administration of rotavirus and picryl chloride challenge, animals of all groups showed a fall in body weight gain. This was correlated with the reduction in diet intake of the infected animals. Parent et al (1984) have reported that both control and vitamin A deficient rats lost weight after infection with a parasite, Schistosoma mansoni. But the non-infected control grew normally. In their study they did not include non-infected vitamin A deficient animals.

This study confirms our previous findings that rotavirus infection causes marked histopathological changes in the small intestine of vitamin A deficient animals compared with controls. Results presented here show that vitamin A deficiency affects the total serum antibody levels specific to rotavirus when given orally.

Oral immunization should provoke local stimulation of IgA antibody and the increase in antibody secretion should be reflected in the serum. There is evidence that in mammalian species, but not in human, a large percentage of serum IgA is dimeric and appears to originate from the mucosal sites (Vaerman and Heremans, 1970). Therefore, our result indicate a defective or decreased antibody production after rotavirus infection. Although it would have been more appropriate to measure the secretory IgA, because of the lack of pure mouse IgA we were unable to demonstrate specific IgA in secretion or serum.

Sirisinha et al (1980) have shown decreased secretory anti-DNP antibody responses in vitamin A deficient rats but the same animals were only marginally affected in terms of their systemic anti-DNP response. This group have also shown that the levels of IgA and IgG in serum were similar in both vitamin A deficient and control rats irrespective of antigen administration. These authors have suggested that vitamin A deficiency does not affect the production of Secretory IgA by the plasma cells but affects the synthesis of the secretory component which makes dimeric IgA refractory to transport into the intestinal lumen. Our study suggests that vitamin A deficiency leads to decreased production of antibody specific to rotavirus antigen, which is at variance with Sirisinha et al (1980). On the other hand, our results could be supported by the study of Smith and Hayes (1987) who have shown that the serum IgG levels of vitamin A deficient mice were significantly lower than controls following intraperitoneal immunization with hemocyanin. Further, total serum immunoglobulins were slightly elevated in pathogen exposed vitamin A deficient mice as compared to controls irrespective of antigen administration. Harmon et al (1963) have shown that vitamin A deficient swine had significantly higher total gamma-globulin fractions as compared to controls, in contrast, the vitamin A deficient swine showed significantly reduced antibody levels against phenolized S. pullorum antigen when compared to controls. Thus the study of Smith and Hayes (1987) and Harmon et al (1963) would support our observations that the vitamin A deficiency leads to impaired

antibody responses following oral challenge.

The impaired antibody response could be the result of a defect in either B-cells, T-cells or both cell types. In order to investigate the T-cell function, we investigated the delayed type hypersensitivity (DTH) response. We observed an impaired DTH response to the skin contact antigen trinitro cholrobenzene (picryl chloride) in the vitamin A deficient mice compared with control and pairfed animals. Thus vitamin A deficiency impairs cell mediated immunity, using the DTH response as an accepted index of cell-mediated immunity (Roitt et al., 1985).

The DTH response involves helper T cells among many other factors and these T cells release lymphokines (Roitt et al, 1985). Therefore, the impaired cell mediated and humoral immunity in our study provides evidence that vitamin A deficiency leads to defective T cell function. Parent et al (1984) have shown that vitamin A deficient rats had significantly reduced antibody titres as compared to controls when infected with a parasite, Schistosoma mansoni. In contrast, they observed a highly specific stimulation of lymphocytes when treated with S.mansoni extract, which implies a normal cellular immune response to this antigen. Their study suggests that vitamin A deficiency specifically impairs B-cell function or alters the balance between the T and B cell arms of the immune response.

Restoring vitamin A levels in the diet one week prior to infection partially restored the capacity of vitamin A deficient animals to mount an antibody response following rotavirus infection, as revealed by histological changes. Although the antibody levels were not significantly different from vitamin A deficient animals, the overall antibody production appeared to have improved since there was no difference observed between refed and controls. These antibody levels could explain why the gut of the vitamin A refed animals showed a more intact histology than vitamin A deficient animals. Refeeding of vitamin A, however, did not result in any improvement in delayed hypersensitivity responses. Since mean antibody levels appeared higher in the refed animals, we assume that helper T-cell function had

returned to normal. Again the impaired DTH response could be due to other factors, such as antigen presenting cells.

In this study, we showed that the relative spleen weights of vitamin A deficient mice were higher compared with control and pairfed animals. These results are again consistant with our previous findings. Since vitamin A deficient animals show an impaired intestinal epithelial barrier, it is possible that the rotavirus or other antigens might pass through the epithelia and entered into the circulation. Thus the vitamin A deficient animals might have an increased level of systemic immune challenge which may not be the case with controls. Therefore, we investigated the effect of vitamin A deficiency on the systemic immune response against rotavirus by intraperitoneal injection.

Our study did not demonstrate any significant change in either antibody or DTH responses due to pairfeeding, which was accompanied by reduced food intake (10-15 % less) in the later part of the study; in disagreement with other reports (Krishnan et al.,1974). Hence, we further investigated the effect of food restriction alone on rotavirus infection.

CHAPTER SEVEN

EFFECT OF VITAMIN A DEFICIENCY ON INTRAPERITONEAL IMMUNIZATION WITH ROTAVIRUS

7.2. Experimental procedure:

The study protocol is presented in figure :7.1. The routine procedures are already described in chapter-2.

7.2.1. Procedure for immunization :

Male Porton mice, 19-21 days old, were initially weight matched and divided into three groups, as control, pairfed and vitamin A deficient. They were supplied with their respective diet as described in chapter-2.2. The weights of the animals were taken weekly and food consumption measured daily.

All animals were kept on their respective diet for 11 weeks and then each group was divided into two subgroups. Animals from one subgroup were immunized with 30 µl/mouse of EDIM rotavirus by intraperitoneal injection and termed "immunized". Animals from the other subgroup were injected with sterile phosphate buffer saline (30 µl/mouse) and termed "non-immunized".

One week after the immunization, the animals were bled by heart puncture and the blood samples were allowed to stand at room temperature for one hour to allow clot formation. After clotting, the blood was centrifuged and the clear serum removed with a pasteur pipette. The serum samples were stored in ependorff tubes at -20°C until analysed. Immediately after the blood had been collected, the carcase of the animals was cut open and the liver, spleen and thymus excised and weighed. The liver was stored at -20°C until analysed for vitamin A content.

7.2.2. Determination of vitamin A:

7.2.2.1. Liver:

Liver vitamin A was determined by the method of Bayfield (1975) with modifications (Section-2.3).

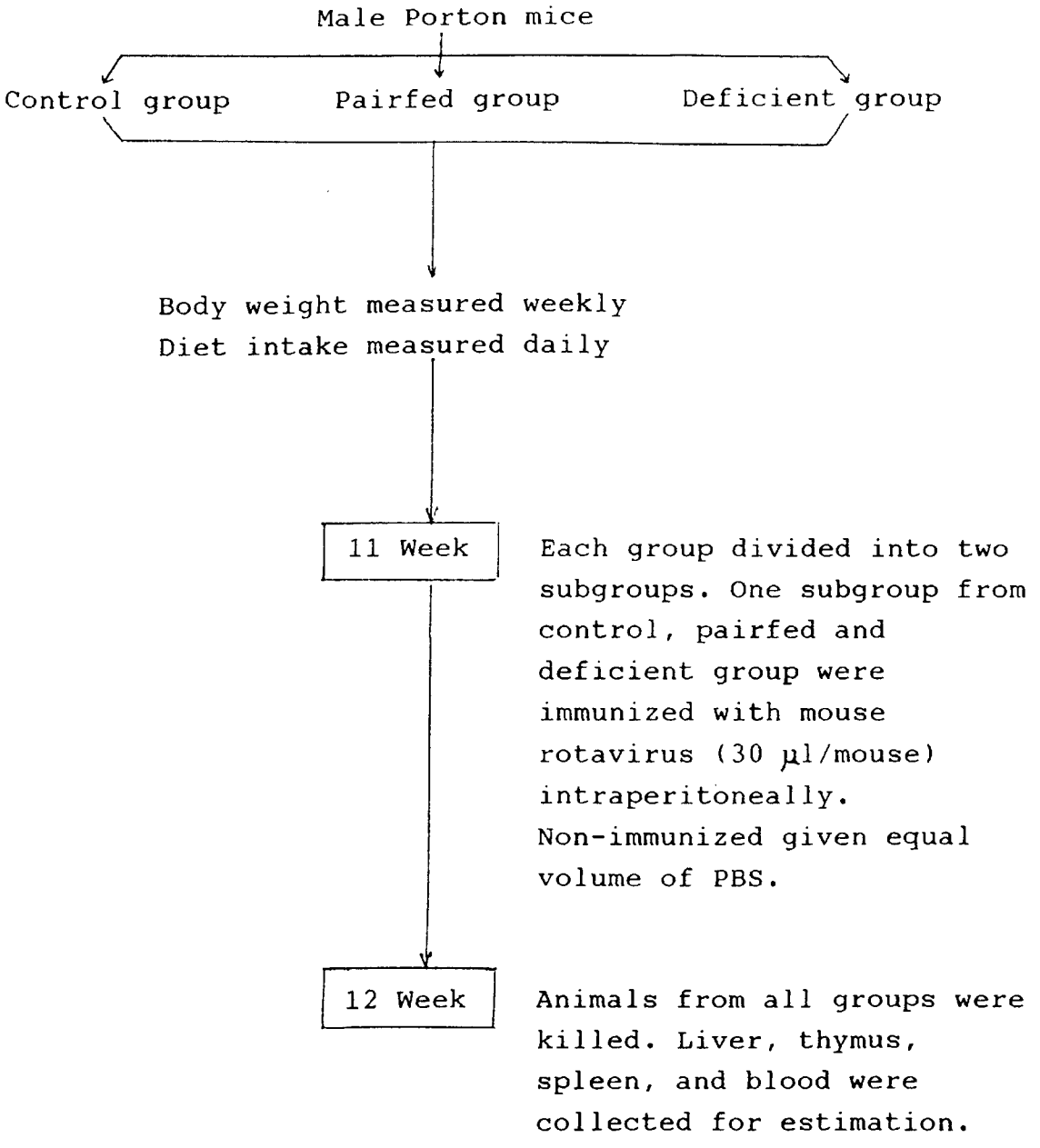

Figure:7.1 Scheme of the procedure for the study of the effect of vitamin A deficiency on the systemic immune response.

7.2.2.2. Serum:

Serum vitamin A was estimated by the high performance liquid chromatography (HPLC) method of Bieri et al (1979) with slight modifications (Section-2.4.2).

7.2.3. Antibody assay:

Total circulating antibody specific to rotavirus antigen was measured using the ELISA method described in section-2.8.2.

7.2.4. Statistical analysis:

The results are expressed as the mean and standard error of the mean for each experimental group. Statistical significance was determined by use of two way analysis of variance (ANOVA) for independent means, except for the antibody results where the Wilcoxon rank sum test for two independent samples was used.

Differences in means values were considered significant when $P<0.05$.

7.3. Results and discussions:

7.3.1. Body weights:

The body weights of all groups throughout the experiment are shown in figure-7.2. The body weight of vitamin A deficient animals reached a plateau at about 9 weeks on the experimental diet and thereafter remained steady. Both the control and pairfed animals gained weight till the end of the study.

After 11 weeks on the diet, vitamin A deficient animals had significantly lower body weights compared with control ($P<0.01$) and pairfed groups ($P<0.05$) irrespective of immunization (univariate level). There was no significant body weight change observed between

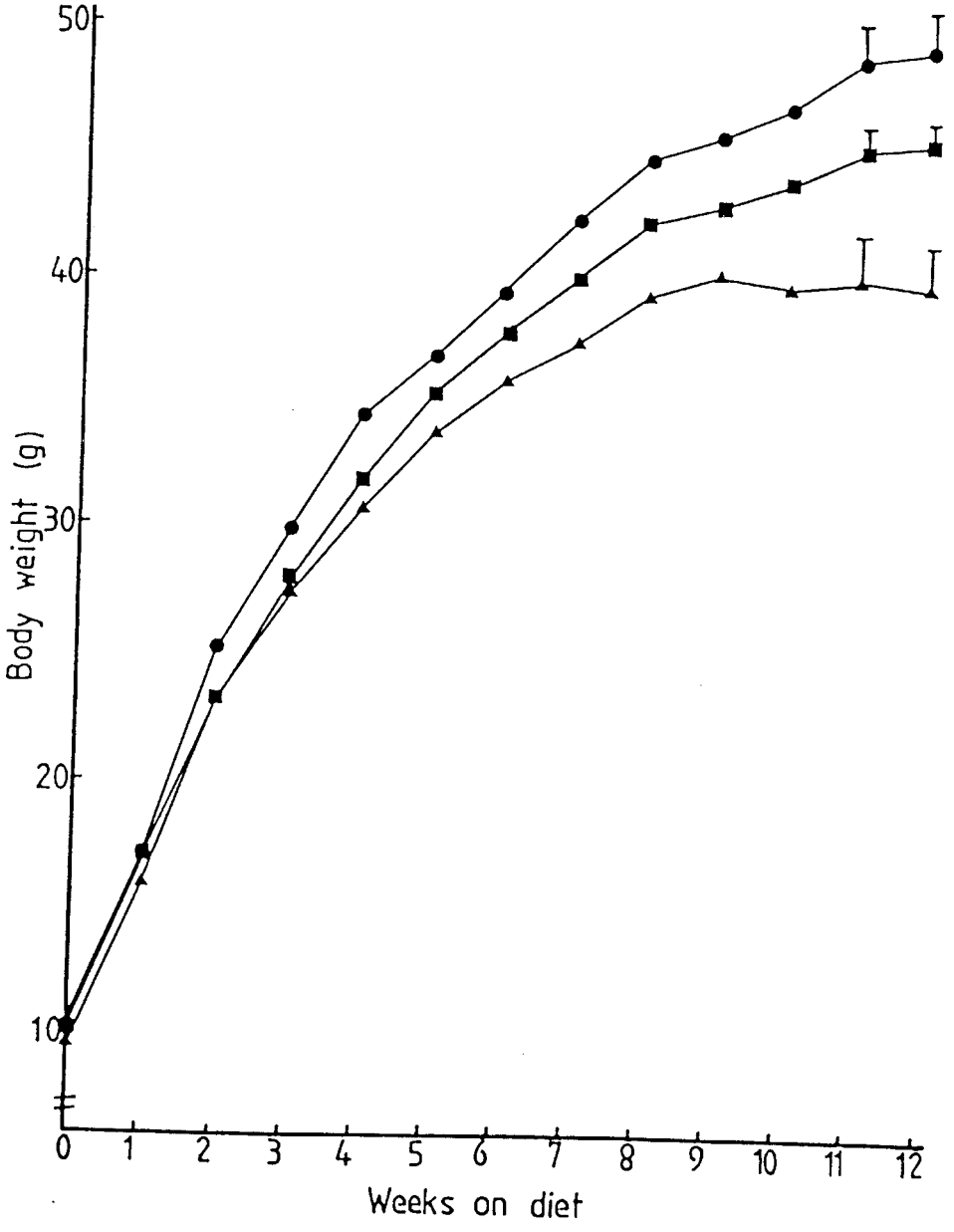

Figure: 7.2 Body weight gain of different groups of animals during the experiment. Control (●—●), pairfed (■—■) and vitamin A deficient (▲—▲) groups. Each point shows the mean of 10 animals. The 11 and 12 week time points display the mean±SEM.

control and pairfed animals. At 12 weeks, the body weights of the vitamin A deficient animals were significantly lower than those of the control ($P<0.01$) and pairfed animals ($P<0.05$) irrespective of immunization (univariate level). There was no significant difference in body weight observed between the control and pairfed animals. There was also no significant change observed between immunized and non-immunized mice. Further no significant changes in the body weights were observed due to the combined effect of diet and immunization.

7.3.2. Diet intake:

The daily diet intakes of the vitamin A deficient and control animals are shown in figure: 7.3. The intake of the vitamin A deficient animals became slightly lower than the control animals after 9 weeks of feeding. There was no change in food intake observed upon immunization.

7.3.3. Organ weights:

7.3.3.1. Liver weight:

The liver weights of the non-immunized and immunized groups are shown in table: 7.1. The total liver weights of the vitamin A deficient animals were significantly lower than those of control ($P<0.01$) and pairfed ($P<0.05$) animals irrespective of immunization (univariate level). There was no significant difference in liver weight between the control and pairfed animals. There was also no significant change observed between immunized and non-immunized animals. Further, there was no significant combined effect observed due to diet and immunization. Our results indicate that rotavirus immunization by the intraperitoneal route does not have any effect on the liver weight over and above that due to vitamin A deficiency. These results parallel those of Takagiand Nakano (1983) where the total liver weight of vitamin A deficient rats was significantly lower

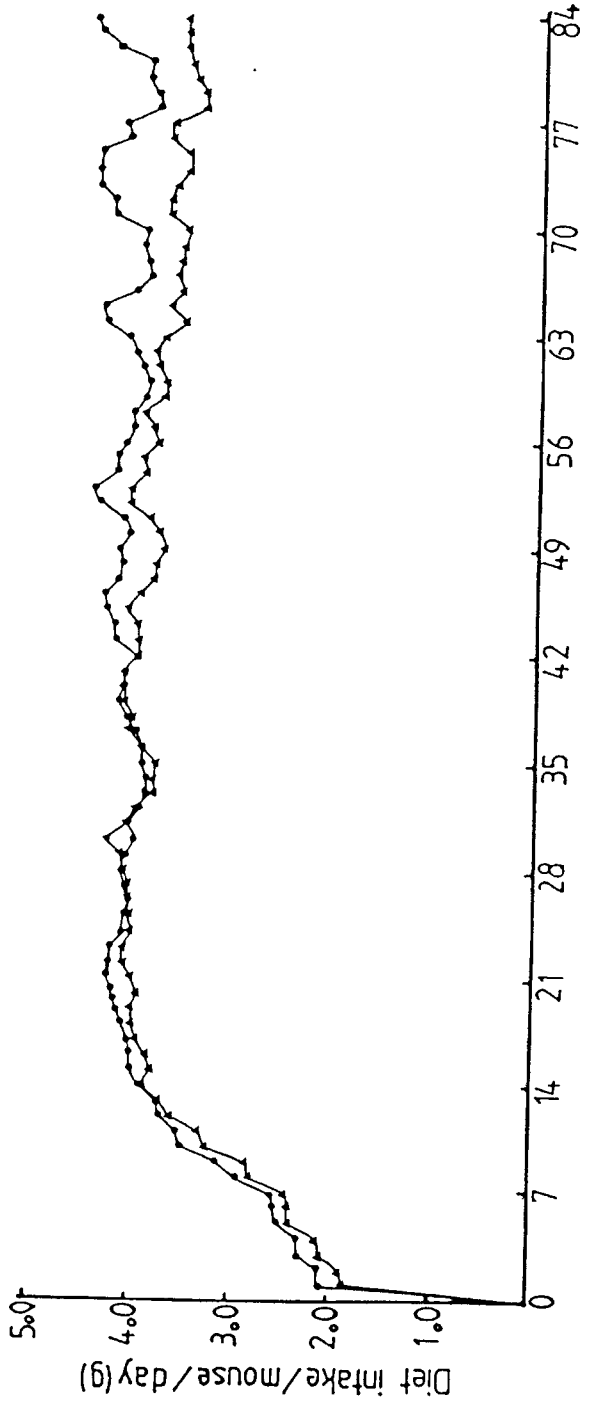

Figure: 7.3. Daily diet intake of the control ad libitum (●—●) and vitamin A deficient animals (▲—▲) throughout the study.

Table: 7.1

Effect of vitamin A deficiency and rotavirus immunization on the liver weight[1]

	Total weight		As % of body weight	
Group	Immunized g	Non-immunized g	Immunized %	Non-immunized %
Control	1.93±0.11	1.82±0.18	3.80±0.1	3.80±0.2
Pairfed	1.79±0.07	1.69±0.06	3.88±0.1	3.70±0.1
Deficient"	1.40±0.14	1.44±0.12	3.59±0.2	3.53±0.1

[1]Results are expressed as the mean±SEM of 5 animals in each group.
"Significantly different from the control ($P<0.01$) and pairfed group ($P<0.05$) irrespective of immunization, when compared for the total weight.

than the control group but no significant difference has observed between the liver weights of non-immunized and SRBC immunized animals.

Liver weights were also expressed as the percentage of body weight (table: 7.1). There was no significant change in the relative liver weight observed between any of the dietary groups irrespective of infection (univariate level). This result is consistant with our previous finding that vitamin A had no effect on relative liver weight. Further there was no difference in relative liver weights of non-immunized and immunized animals.

7.3.3.2. Spleen weight:

The spleen weights of both the non-immunized and immunized animals are shown in table: 7.2. The total spleen weights of the vitamin A deficient animals were similar to those of control and pairfed animals. There were no significant changes in spleen weight observed following immunization. Takagi and Nakano (1983) have shown that when rats were immunized with SRBC the total spleen weights of the control rats were significantly higher than those of the corresponding vitamin A deficient rats, this is in disagreement with our findings with rotavirus.

The relative spleen weights of the vitamin A deficient animals were significantly higher than those of control animals irrespective of immunization ($P<0.05$, univariate level). Again, when we performed two way analysis of variance, the relative spleen weights of the non-immunized animals were found to be significantly higher than that of immunized animals irrespective of dietary treatment. No significant interaction was observed between diet and immunization. Relative spleen weights of control and pairfed groups were similar.

7.3.3.3. Thymus weight:

The thymus weights of both the immunized and non-immunized groups are shown in table: 7.3. The total thymus weights of the

Table: 7.2

Effect of vitamin A deficiency and rotavirus immunization on the spleen weight[1]

	Total weight		As % of body weight	
Group	Immunized mg	Non-immunized mg	Immunized %	Non-immunized"" %
Control	87.3±4.4	105.2±9.0	0.17±0.01	0.22±0.02
Pairfed	97.8±5.3	93.6±5.0	0.21±0.01	0.21±0.01
Deficient"	82.0±4.7	111.0±16.0	0.21±0.01	0.27±0.03

[1]Results are expressed as the mean±SEM of 5 animals in each group.
"Significantly different from the control group ($P<0.05$) irrespective of immunization, when compared for the relative weight.
""Significantly different from the immunized group ($P<0.05$) irrespective of dietary treatment, when compared for the relative weight.

Table: 7.3

Effect of vitamin A deficiency and rotavirus immunization on the thymus weight[1]

Group	Total weight		As % of body weight	
	Immunized mg	Non-immunized mg	Immunized %	Non-immunized %
Control	53.2±2.4	51.3±2.9	0.105±0.003	0.108±0.004
Pairfed	50.4±3.7	50.0±1.5	0.109±0.007	0.109±0.003
Deficient"+	35.3±4.7	40.7±3.4	0.090±0.008	0.100±0.004

[1]Results are expressed as the mean±SEM of 5 animals in each group.
"Significantly different from the control and pairfed group ($P<0.01$) irrespective of immunization, when compared for the total weight.
+Significantly different form the pairfed group ($P<0.05$) irrespective of immunization, when compared for the relative weight.

vitamin A deficient animals were significantly lower than those of control and pairfed animals irrespective of immunization ($P<0.01$, univariate level). There was no significant change observed due to the immunization. Further, there was no interaction observed between diet and immunization. We observed no significant difference between control and pairfed animals. These results suggest that total thymus weight is affected by the vitamin A deficiency alone or in combination with low food intake.

The relative thymus weight of the vitamin A deficient animals was significantly lower than those of the pairfed animals irrespective of immunization ($P<0.05$). There was no significant change observed due to immunization. Further there was no significant interaction between the diet and immunization.

7.3.4. Vitamin A levels:

7.3.4.1. Liver vitamin A:

Liver vitamin A content of both the non-immunized and immunized animals are shown in table: 7.4. The total liver vitamin A content of the vitamin A deficient group was significantly lower than the control and pairfed animals irrespective of immunization ($P<0.01$, univariate level). There was no significant difference observed between control and pairfed animals. No significant difference of liver vitamin A content was observed between immunized and non-immunized animals. Further, there was no interaction observed between diet and immunization.

7.3.4.2. Serum vitamin A:

The serum vitamin A levels of both the immunized and non-immunized animals are shown in table: 7.5. The serum vitamin A levels of the vitamin A deficient group were significantly lower than any other dietary groups irrespective of immunization ($P<0.01$). There

Table: 7.4

Effect of vitamin A deficiency and rotavirus immunization on the liver vitamin A[1]

Group	Immunized µg/liver	Non-immunized µg/liver
Control	229.7±8.2	218.9±7.6
Pairfed	205.6±8.0	207.6±10.0
Deficient"	0.4±0.1	0.5±0.2

Table: 7.5

Effect of vitamin A deficiency and rotavirus immunization on the serum vitamin A[1]

Group	Immunized µg/100ml	Non-immunized µg/100ml
Control	43.7±3.0	42.8±3.8
Pairfed	43.5±3.6	41.7±2.4
Deficient"	2.8±0.3	2.4±0.3

[1]Results are expressed as the mean±SEM of 5 animals in each group.

"Significantly different from the control and pairfed group ($P<0.01$) irrespective of immunization.

was no significant difference observed between control and pairfed groups. Immunization did not alter serum vitamin A levels. Finally there was no interaction observed between the diet and rotavirus immunization.

7.3.5. Antibody levels specific to rotavirus:

The total antibody levels specific for rotavirus for both non-immunized and immunized animals are shown in table: 7.6. In the case of non-immunized animals the antibody levels were measured by using pooled serum from each group. The antibody titre of the non-immunized animals served as the background antibody level, since these mice already had rotavirus antibody in their serum. In the immunized animals, the antibody titres were measured by duplicate reading of each serum sample. The total serum antibody levels specific to rotavirus in the immunized animals were only marginally higher than those of non-immunized animals. In order to compare the data between the immunized groups, Wilcoxon rank sum test was applied and there was no significant difference in the antibody levels observed between vitamin A deficient, control or pairfed groups.

In the present study, intraperitoneal immunization with EDIM rotavirus has failed to provoke a specific antibody response. Therefore, it is difficult to asses the role of vitamin A in systemic antibody production.

7.4 Conclusions:

The present study was designed to investigate the role of vitamin A deficiency on the systemic immune response. The protocol of the present study was exactly the same as the previous experiment, except for the route of immunization. In the previous study antibody levels specific to rotavirus were investigated after oral challenge with 30 µl/mouse EDIM rotavirus. In the present study the same dose of EDIM rotavirus was injected intraperitoneally to provoke the systemic

Table: 7.6

Effect of vitamin A deficiency on the serum antibody levels specific to rotavirus[1]

Group	Non-Immunized* Units	Immunized** Units
Control	170.0	195.8±37.4
Pairfed	138.0	227.2±27.5
Vitamin A deficient	159.0	229.6±46.4

[1]Results are expressed as the units of anti rotavirus activity extrapolated from the standard curve of the standard serum (see chapter-2.8.2).

*Shows the mean antibody levels of the pooled serum of 5 animals in each group.

**Shows the mean±SEM of 5 samples in each group.

antibody response.

The experimental design produced vitamin A deficient animals as judged by the body weight, liver vitamin A and serum vitamin A which is consistant with our previous study. However, the intraperitoneal immunization failed to give any significant changes in the total serum antibody levels specific to rotavirus in the vitamin A deficient mice when compared to that of pairfed and control mice. Indeed the intraperitoneal injection of EDIM rotavirus (30 µl/mouse) was unable to produce sufficient antibody in any of the groups. Therefore, at present it is difficult to make any definite conclusion of the effect of vitamin A deficiency on the systemic antibody production to rotavirus.

The failure of antibody production could be due to several factors. Most important among them is the dose of virus used for immunization. In the oral challenge 30 ul/mouse EDIM rotavirus was used since this amount was just sufficient to cause diarrhoea in the infant mice (Starkey et al., 1986) and was able to produce significant amount of antibody (chapter-six). Since the antigen used is a replicative antigen and gut is the site of replication, it seems that after oral challenge antigens replication was sufficient to provoke an antibody response. Whereas in the intraperitoneal immunization the rotavirus was not able to replicate and thus the dose was insufficient to provoke an antibody response.

CHAPTER EIGHT

EFFECT OF MODERATE FOOD RESTRICTION TO ROTAVIRUS INFECTION

CHAPTER EIGHT

8.1. Introduction:

Previous sections of this thesis have delt with the effect of vitamin A deficiency on rotavirus infection and immunization. It was observed that vitamin A deficient mice were highly susceptible to rotavirus infection as judged by the histopathology of the small intestine (chapter-five) and had impaired immunity as the serum antibody specific to rotavirus was significantly lower than that of control and pairfed animals (chapter-six). It was evident from these studies that the pairfed animals had normal resistance and were capable of producing similar antibody levels following infection with EDIM rotavirus as controls. Others have claimed that pairfed rats are equally susceptible to oral infection with Salmonella typhimurium as compared to vitamin A deficient rats (Kligler et al.,1945). Krishnan et al (1974) have shown that pairfed rats had the same haemolysin and haemagglutinin titres as in vitamin A deficient rats following immunization with sheep RBC. Clearly these results contradict our findings. However, towards the end of the 12 week experimental period the pairfed animals in the present study had eaten only 10-12 % less food compared with controls. In order to justify our results, therefore it was of importance to evaluate the role of reduced food intake in rotavirus infection. In this study, reduced food intake means the reduction of all components of a balanced diet. Therefore, the animals on reduced food will receive fewer calories as well as a reduced intake of vitamins and minerals when compared with controls.

Further, it is necessary to establish that the diet which we have developed for the induction of vitamin A deficiency is able to provide all other dietary requirements adequately, so that it becomes balanced only by adding vitamin A. We therefore decided that it would be of interest to compare our diet with a commercial diet which is designed for the mouse.

The present study was designed therefore to investigate the effect of reduced food intake on the rotavirus infection by oral challenge and also to investigate the immune response, both humoral and cellular. Further, the diet in the present study was compared with a commercial diet in respect to body weight gain and immune response.

8.2. Experimental procedure:

The study protocol is presented in figure :8.1. The routine procedures are already described in chapter-2.

8.2.1. Procedure for immunization :

Male Porton mice, 28 days old, were initially weight matched and divided into four groups. Group-1 (Com. diet) was fed commercial diet given ad libitum and the composition of this diet is shown in table:8.1. Group-2 (Control) was fed control diet ad libitum and the composition of this diet is already shown in chapter-2.2. Group-3 (Con-15) and group-4 (Con-30) were fed 15 and 30 % less control diet than the group-2 animals had eaten on the previous day. Each group had two subgroups, one subgroup termed "non-infected" was kept in a conventional room. The other subgroup termed "infected" was kept in an isolation room from the start of the experiment.

The animals were housed in groups of two or three in plastic cages. The room temperature was 20±2°C, humidity 50±5 % and 12 hours lighting schedule per day. The weights of the animals were taken weekly and food consumption measured daily.

8.2.1.1. Antibody production:

At day 42, animals from the "infected" group were dosed with 30 µl/mouse of EDIM rotavirus orally. Animals from the non-infected group were dosed with 30 µl/mouse of phosphate buffer saline (PBS). 7 days post-dosing blood samples were collected.

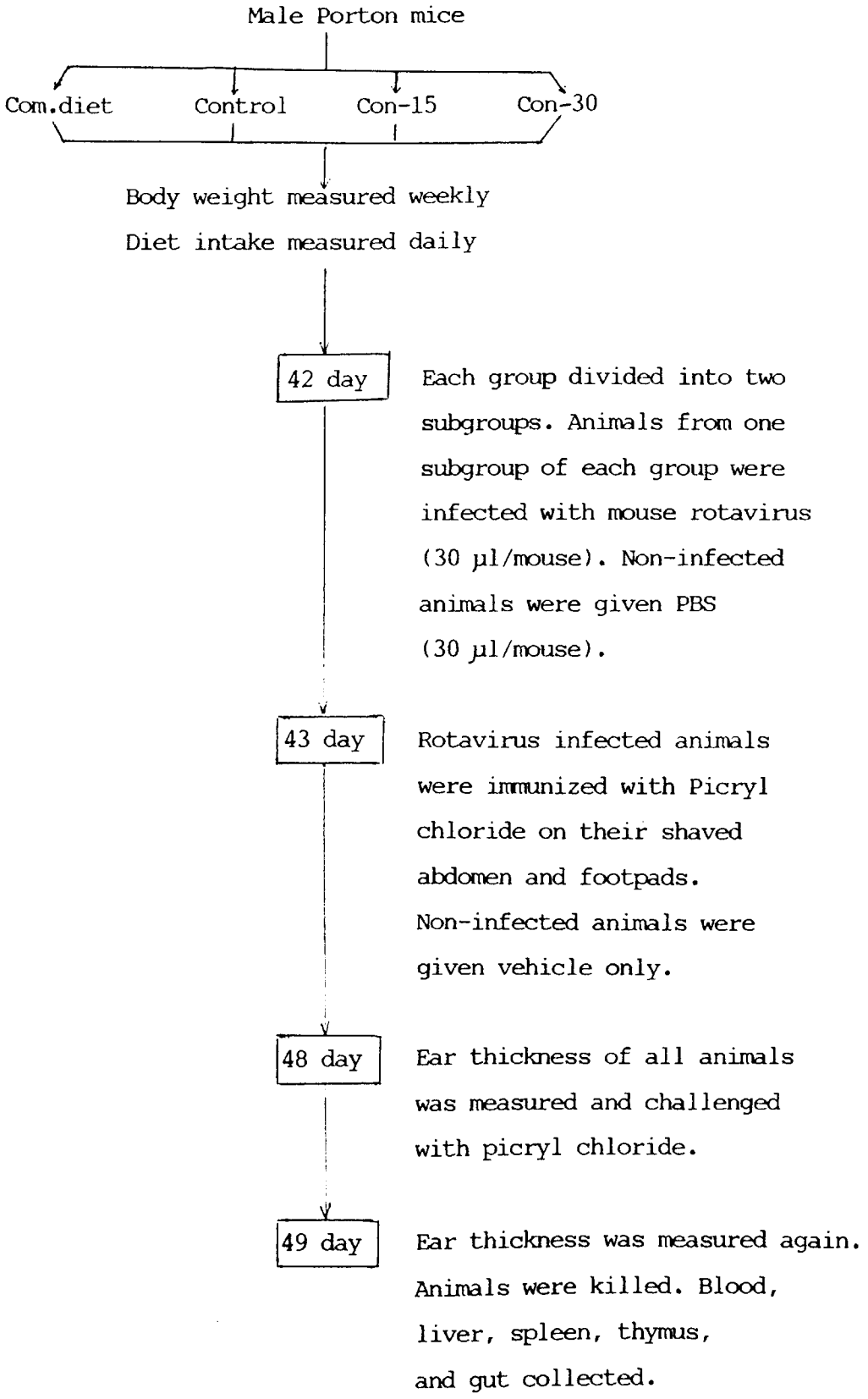

Figure: 8.1 Scheme of the procedure for the study of the effect of moderate food restriction and rotavirus infection.

Table: 8.1

Composition of Commercial diet[1]

Proximate analysis	as%	Trace Element added	
Carbohydrate	56.33	Manganese	25 ppm
Crude protein	18.30	Copper	7 ppm
Crude oil	2.90	Cobalt	0.4 ppm
Crude fibre	3.50	Iron	30 ppm
Calcium (as Ca)	0.80	Iodine	1.3 ppm
Phosphorus (as P)	0.60	Magnesium	102 ppm
Salt	0.70		

Amino acids (as % of feed)		Vitamin Added (per kg)	
Threonine	0.6	Vitamin A	8000 I.U.
Glycine	0.9	Vitamin D_3	1000 I.U.
Valine	0.8	Vitamin B_2	8 mg
Cystine	0.2	Nicotinic acid	50 mg
Methionine	0.3	Pantothenic acid	12 mg
Isoleucine	0.7	Vitamin B_{12}	12 ug
Leucine	1.4	Vitamin E	60 I.U.
Tyrosine	0.6	Vitamin K	10 mg
Phenylalanine	0.8	Folic acid	10 mg
Lysine	1.0	Choline chloride	200 mg
Histidine	0.4	Vitamin B_1	4 mg
Arginine	1.2	Vitamin B_6	6 mg
Tryptophan	0.2		

[1]Obtained from SDS, England.

8.2.1.2. Delayed hypersensitivity test:

For the demonstration of delayed hypersensitivity, on day 43 (one day after rotavirus dosing) the animals in the infected groups were immunized with 150 µl and 50 µl of 5 % Picryl chloride in acetone:alcohol (1:9 vol) on the shaved abdomen and to the four foot pads. Non-infected animals received the same amount of acetone and alcohol solution on the shaved abdomen and to the four foot pads. 24 hours before killing the ear thickness of all animals was measured by micrometer followed by challenged with 1 % picryl chloride solution in acetone: olive oil (1:9 vol) on both ears. On day 49, or one week post-dosing, the ear swelling of all animals was measured.

The animals were bled by heart puncture and blood samples were allowed to stand at room temperature for one hour to allow clot formation. After clotting, the blood was centrifuged and the clear serum removed with a pasteur pipette. The serum samples were stored in ependorff tubes at -20°C until analysed. Immediately after the blood was collected, the carcase of the animals was cut open and the liver, spleen, and thymus were excised and weighed. The liver was stored at -20°C until required for analysis. Spleen, thymus and a representative portion of small intestine were fixed in 10 % buffered formalin solution for histological analysis.

8.2.2. Determination of vitamin A:

8.2.2.1. Liver:

Liver vitamin A was determined by the method of Bayfield (1975) with the modifications (Section-2.3).

8.2.3. Histological study:

The histology of the spleen, thymus, and gut was examined by

the method described in section-2.5.

8.2.4. Antibody assay:

Total circulating antibody specific to rotavirus antigen was measured by using the ELISA method described in section-2.8.2.

8.2.5. Cell mediated immunity:

Delayed type hypersensitivity response was measured by using the method described in section-2.9.

8.2.6. Statistical analysis:

Results are expressed as the mean and standard error of the mean. Differences between groups were evaluated by using 2-way analysis of variance (ANOVA) for independent means for the parametric data and Wilcoxon rank sum test for the non-parametric data.

Differences of the means were considered significant when $P<0.05$.

8.3. Results and discussions:

8.3.1. Body weights:

The body weight of all groups of non-infected and infected animals throughout the experiment is shown in figure-8.2. The growth of the non-infected animals of all groups continued until the end of the study. Both the con-15 (15 % less food intake) and con-30 (30 % less food intake) groups gained less weight than the control group. The animals in the infected groups grew at a similar rate to corresponding non-infected groups before infection. Six weeks after feeding, the average body weight of the con-15 and con-30 groups were 91 % and 80 % that of control group respectively. The body weight of

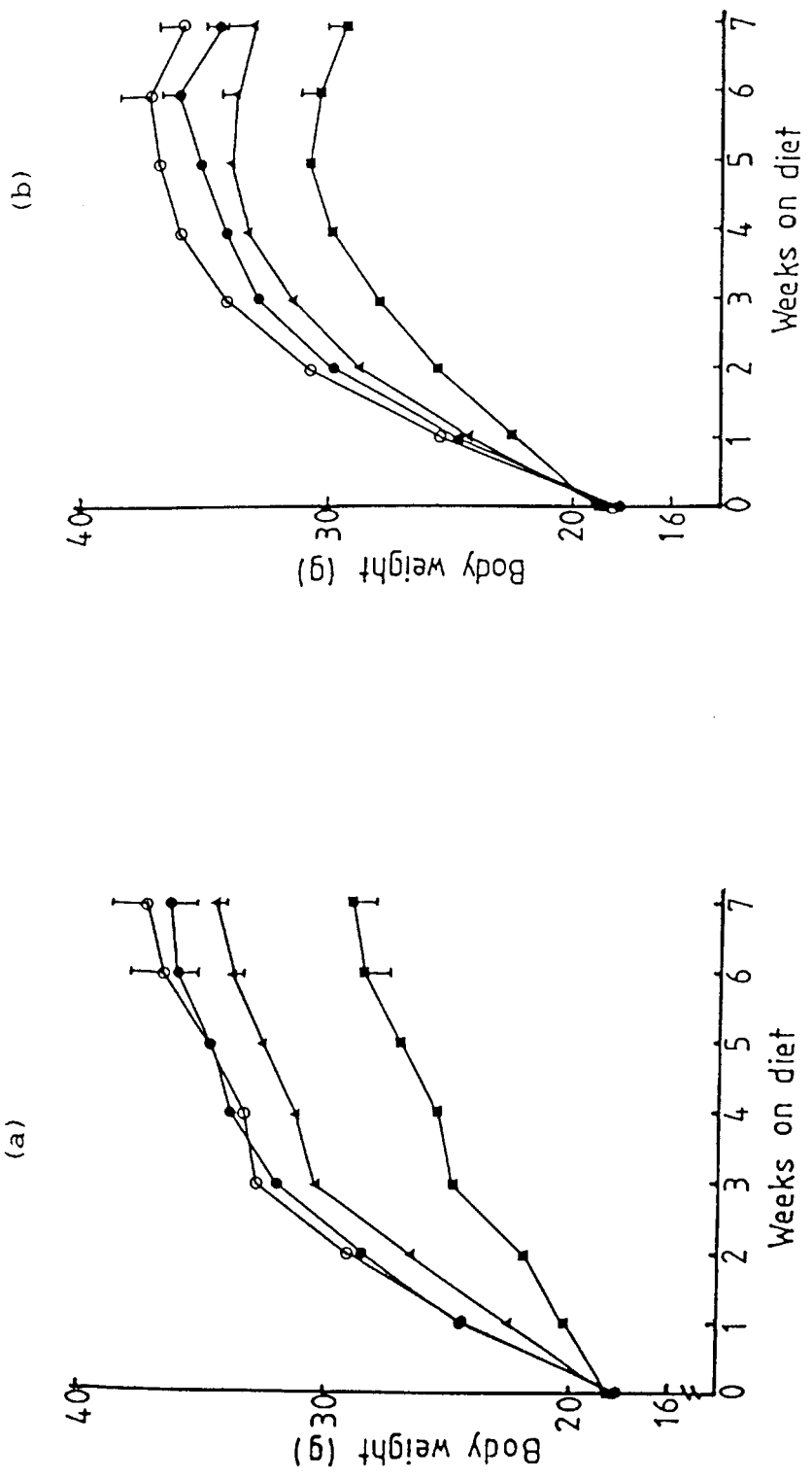

Figure: 8.2 Body weight gain of (a) non-infected and (b) infected animals during the moderate food restriction experiment. Com.diet (●—●), control (○—○), con-15 (▲—▲) and con-30 (■—■). Each point shows the mean of 5 animals. The 11 and 12 week time points display the mean±SEM.

con-30 was 87 % that of the con-15 group. After infection with rotavirus and picryl chloride challenge, a sharp fall in the body weight of all infected animals was observed.

When we applied 2 way analysis of variance for the determination of statistical significance , the body weights of the con-15 group appeared significantly lower than those of the control group after six weeks ($P<0.001$) and seven weeks ($P<0.05$) of feeding irrespective of infection (univariate level). On the other hand, the body weights of the con-30 group were significantly lower than any other dietary group irrespective of infection (univariate level) both at six and seven week time point ($P<0.001$). These results indicate that the restriction in food intake had profoundly impaired normal growth. Reduced food intake, protein-calorie malnutrition or protein deficiency has been commonly observed to reduce body weight gain (McAnulty and Dickerson, 1973; Brown and Guthrie,1968; Badger et al., 1972). In the present study there was no significant interaction observed between the diet and infection at either six or seven weeks.

There was no significant difference in the body weights observed between the control and com.diet group. From this result it is evident that the diet which we have developed for the vitamin A deficient model supports growth as effectively as a balanced commercial mouse diet.

8.3.2. Diet intake:

The daily diet intakes of control and com.diet group are shown in figure: 8.3 and 8.4 for the non-infected and infected animals respectively. The intake of the non-infected animals of both the control and com.diet group were similar throughout the study. After infection and immunization at 6 weeks, animals from all infected groups showed a sharp fall in food intake for the first few days and thereafter the intake returned to the previous level, in figure: 8.4. As before, we cannot attribute the reduced food intake to picryl chloride treatment alone or to the mixed antigen challenge.

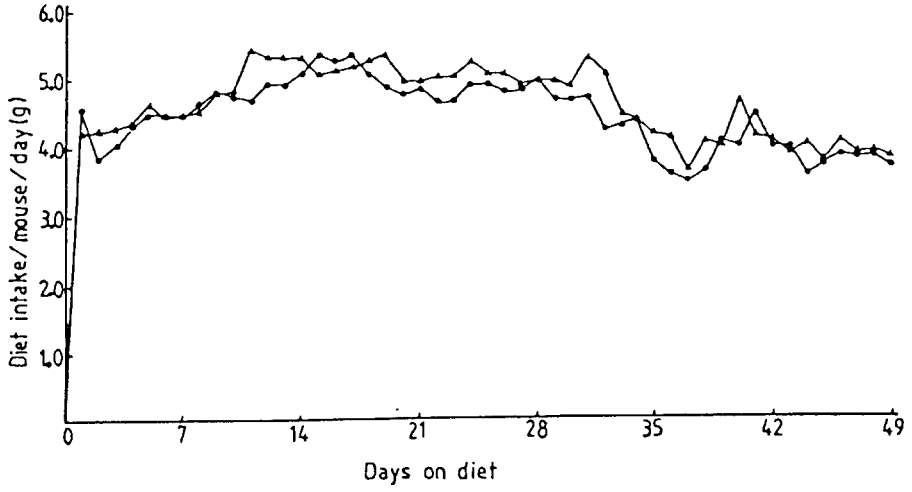

Figure: 8.3 Daily diet intake of the non-infected com.diet (▲—▲) and control (●—●) groups throughout the study.

Figure: 8.4 Daily diet intake of the infected com.diet (▲—▲) and control (●—●) groups throughout the study.

8.3.3. Organ weights:

8.3.3.1. Liver weight:

The liver weights of the non-infected and infected groups are shown in table: 8.2. Total liver weights of con-30 animals were significantly lower than those of any other dietary group irrespective of infection ($P<0.01$, univariate level). There was no significant difference in liver weight observed between any other dietary groups . No significant difference was found between infected and non-infected groups. Further there was no interaction observed between the diet and infection. These results show that a reduced food intake of up to 15 % of control intake does not affect total liver weight. It is clear therefore that the body weight is affected to a greater extent than liver weight when animals have eaten 15 % less diet than controls. The present study confirms our previous result in that the total liver weights of pairfed animals are similar to those of controls. Finally, infection does not have any effect on the liver weight over and above that due to low food intake, this is also consistant with our previous findings.

The relative liver weights (% of body weight) of the con-30 group were significantly lower than that of control group irrespective of infection ($P<0.05$, univariate level). There was no significant difference in the relative liver weights observed between any other dietary groups. This result indicates that the liver weight is affected more than the body weight when animals have eaten 30 % less food. Badger et al (1972) have shown that when young Sinclair (S-1) miniature swine were weaned at day 5 with either a 5% protein diet or a reduced food intake (pairfed to the 5 % protein diet group fed 20 % protein diet) and fed for 8 weeks at this level they had a significantly lower total liver weight compared with the control ad libitum group (fed 20% protein diet), although the relative liver weights of all the three groups were similar. This is not in agreement with our results where 30 % less food intake caused a significant

Table: 8.2

Effect of moderate food restriction and rotavirus infection on the liver weight[1]

	Total weight		As % of body weight	
Group	Infected g	Non-infected g	Infected %	Non-infected %
Com.diet	1.69±0.06	1.63±0.10	4.9±0.2	4.5±0.2
Control	1.68±0.05	1.80±0.12	4.7±0.2	4.8±0.2
Con-15	1.58±0.10	1.49±0.10	4.8±0.2	4.3±0.3
Con-30[*+]	1.26±0.03	1.20±0.05	4.3±0.1	4.1±0.2

[1]Results are expressed as the mean±SEM of 5 animals in each group.
[*]Significantly different from any other dietary group ($P<0.01$) irrespective of infection, when compared for the total weight.
[+]Significantly different from control group ($P<0.05$) irrespective of infection, when compared for the relative weight.

change in relative liver weight. However the pairfed animals in their study had eaten about 50 % less food in the first half of the experiment and 75 % less in the rest of the period compared with control ad libitum animals. In these animals the rate of liver weight gain was proportional to the body weight gain, thus relative liver weights were not affected due to reduced food intake. Brown and Guthrie (1968) have shown that severe undernutrition, both protein and calorie restriction, for three weeks in infant rats significantly lowered total liver weight as compared to well fed infants. The relative liver weight were similar.

8.3.3.2. Spleen weight:

The spleen weights of both the non-infected and infected animals are shown in table: 8.3. Total spleen weights of the infected animals were significantly higher than those of the non-infected animals irrespective of dietary treatment ($P<0.01$, univariate level). The total spleen weights of con-30 were significantly lower than those of the com.diet group irrespective of infection. However, the total spleen weights of both the con-15 and con-30 group appeared normal when compared to those of the control ad libitum group. In contrast, Bell et al (1976) have shown that protein-calorie malnourished mice had a significantly reduced spleen weight as compared to controls, although these animals had eaten only 4 % protein diet in addition to reduced food intake. Kenney et al (1968) have also shown that adult rats fed a low protein diet for 5 weeks had spleen weights of about 60 % of control animals. Both of these studies have used very low protein diets and have in fact demonstrated the effect of protein-deficiency rather than low food intake on spleen weight.

When relative spleen weights were compared statistically, the infected animals showed significantly higher values than the non-infected animals irrespective of any dietary treatment ($P<0.01$, univariate level). There was no significant difference in the relative spleen weight due to reduced food intake irrespective of infection.

Table: 8.3

Effect of moderate food restriction and rotavirus infection on the spleen weight[1]

	Total weight		As % of body weight	
Group	Infected* mg	Non-infected mg	Infected* %	Non-infected %
Com.diet	111.4±5.3	97.1±4.0	0.32±0.02	0.27±0.01
Control	101.1±5.6	91.3±9.1	0.28±0.02	0.24±0.02
Con-15	91.8±11.7	81.3±4.5	0.28±0.03	0.24±0.01
Con-30**	96.5±8.4	68.3±5.6	0.33±0.03	0.23±0.02

[1]Results are expressed as the mean±SEM of 5 animals in each group.

*Significantly different from the corresponding non-infected groups ($P<0.01$) irrespective of any dietary treatment.

**Significantly different from Com.diet group ($P<0.05$) irrespective of infection, when compared for the total liver weight.

There was also no significant interaction between diet and infection either on the total or relative spleen weights.

8.3.3.3. Thymus weight:

The thymus weights of both the infected and non-infected groups are shown in table: 8.4. Total thymus weights of the infected group were significantly lower than those of the non-infected group irrespective of any dietary treatment ($P<0.01$, univariate level). No significant interaction was observed between diet and infection. Further there was no significant difference observed between any of the dietary groups irrespective of infection. These results suggest that total thymus weight is not affected by the reduction of food intake alone and is in agreement with our previous findings, where we have shown that pairfed animals showed similar thymus weights to controls. Other investigators have also shown that total thymus weight was not affected by the severe undernutrition for 3 weeks in infant rats (Brown and Guthrie, 1968). In contrast, when McAnulty and Dickerson (1973) fed a reduced amount of food so that weanling rats grew at a constant rate, they found significantly lower total and relative thymus weights after 4 weeks when compared with control ad libitum fed animals.

When we considered relative thymus weight, the infected animals had a significantly lower weight than those of the non-infected animals irrespective of dietary treatment ($P<0.01$, univariate level). Diet alone did not have any effect on relative thymus weight. There was also no significant interaction observed between the diet and infection. The reduced thymus weights of the infected animals observed in the present study is difficult to explain but could be due either to the picryl chloride treatment alone or to the cumulative effect of both antigens employed in the study.

8.3.4. Vitamin A levels:

Table: 8.4

Effect of moderate food restriction and rotavirus infection on the thymus weight[1]

	Total weight		As % of body weight	
Group	Infected* mg	Non-infected mg	Infected* %	Non-infected %
Com.diet	36.9±2.1	56.8±4.8	0.108±0.01	0.156±0.01
Control	30.2±1.4	60.4±3.6	0.084±0.005	0.161±0.005
Con-15	25.5±4.9	54.0±2.7	0.076±0.01	0.156±0.01
Con-30	25.9±2.4	54.3±2.6	0.088±0.01	0.186±0.01

[1]Results are expressed as the mean±SEM of 5 animals in each group.
*Significantly different from the corresponding non-infected groups ($P<0.01$) irrespective of any dietary treatment.

8.3.4.1. Liver vitamin A :

The liver vitamin A content of both non-infected and infected animals is shown in table: 8.5. The liver Vitamin A levels of the infected animals were significantly higher than those of the non-infected animals independent of any dietary treatment ($P<0.01$, univariate level). One possible explanation might be that the synthesis of the negetive acute phase reactant, RBP, is reduced in the face of infection, thereby limiting the mobilisation of vitamin A (Golden,M.H.N.1982). The liver vitamin A levels of the com.diet group were significantly higher than any other dietary group irrespective of infection ($P<0.01$). This higher liver vitamin A level can be attributed to the vitamin A content in the com.diet. Since com.diet contained 8000 I.U./kg diet whereas our diet contained 4000 I.U./kg diet. Further, there was no significant interaction observed between the diet and infection. In the present study low food intake groups had similar liver vitamin A levels as that of control ad libitum group. Zaklama et al (1972) have described the effects of protein-calorie malnutrition on liver vitamin A levels and their results did not show any significant change in the liver vitamin A levels when compared to that of their control ad libitum group. These authors used weanling rats fed a 3 % protein diet for 8 weeks with an overall food intake over the entire period of approximately 30% less than the control ad libitum animals. Our results are consistant with their finding. They suggest a high rate of utilization of vitamin A in the high protein fed ad libitum groups.

8.3.5. Histological study:

8.3.5.1. Spleen:

The histology of the spleen in both the non-infected and infected groups was examined by light microscopy. Both the red pulp

Table: 8.5

Effect of moderate food restriction and rotavirus infection on the liver vitamin A[1]

Group	Infected* µg/liver	Non-infected µg/liver
Com.diet**	374.2±18.7	329.9±14.8
Control	125.0±4.5	126.8±11.5
Con-15	135.1±1.6	128.5±3.5
Con-30	123.3±3.4	116.0±3.4

[1]Results are expressed as the mean±SEM of 5 animals in each group.

*Significantly different from the non-infected group ($P<0.05$) irrespective of any dietary treatment.

**Significantly different from any other dietary group ($P<0.01$) irrespective of infection.

and the white pulp areas of all dietary groups appeared normal. In the white pulp, the periarteriolar lymphoid sheaths (PALS) were seen in various planes of section. There was no significant difference observed in the B cells areas, due either to the dietary treatment or to rotavirus infection. T cells areas also appeared normal in all groups. Although the spleen weights of the infected animals were significantly larger than the non-infected animals, it was not possible to detect any histological changes.

8.3.5.2. Thymus:

The histology of the thymus of both the non-infected and infected groups was examined under light microscopy. The outer cortical area in all groups irrespective of infection was found to be normal and no involution was observed. The medulla of all dietary groups was also found to be normal in appearance in both the non-infected and infected animals. The area of the medulla of all the infected animals looked smaller than in the non-infected animals, although it is very difficult to compare the cortical or medullary areas between different sections. In practice it is not possible to collect the same relative area of thymus for the histological examination for all groups of mice. As we had seen a relative fall in thymus weight in the infected animals of all groups as compared to non-infected animals, we were expecting the infected thymus to be less cellular. However we could not reliably compare the area of the medulla or cortex of the deficient animal with the controls. Bell et al (1976) have investigated the effect of dietary protein restriction on the weanling mice fed a 4 % protein diet for 4 weeks. Their results show a loss of cortex and apparently reduced medullary mass. However they were not able to accurately quantify these changes and suggested a normal structure of thymus appart from an apparent loss of mature lymphocytes. Their animals, when fed 4 % protein diet had eaten less diet than that of the controls fed 20 % protein diet and the experimental status therefore reflects the effects of severe

protein-calorie malnutrition. The diet of the present experiment provided 20 % protein but was low in total food intake.

8.3.5.3. Gut:

The histology of the small intestine (middle portion) of both the infected and non-infected groups was examined under the light microscope. The villi of all mice examined appeared normal and intact. After rotavirus infection, there were no significant changes observed in any of the dietary groups. This finding indicates that the mice fed at up to 30 % less food intake than controls, for 7 weeks are as capable of protecting their gut from rotavirus infections as control animals.

8.3.6. Serum antibody levels:

The total serum antibody levels, specific to rotavirus, of each of the dietary groups are shown in table: 8.6. The data showing units of rotavirus antibody was calculated from the standard curve obtained from the standard rotavirus serum (Mouse anti rotavirus) in each ELISA plate. In the case of non-infected animals, the antibody units show only the mean of each of the groups. However, the serum samples of each group were pooled together and then the antibody levels measured. After rotavirus infection, there was substantial increases in the serum antibody observed in all the dietary groups. Although both the con-15 and con-30 groups showed apparently higher antibody levels than control and com.diet groups, this did not reach statistical significance. This result indicates that a reduced food intake for 7 weeks does not in itself impair the serum antibody levels following oral challenge of rotavirus, when compared to that of control ad libitum fed animals. Further, the antibody levels of the com.diet group appeared similar to the control group. Kenney et al (1968) have shown that when adult male rats are fed a low protein diet for 5 weeks they show low serum antibody levels against SRBC given

Table: 8.6

Effect of moderate food restriction on the total serum antibody levels specific to rotavirus[1]

Group	Non-infected* Units	Infected** Units
Com.diet	170.0	820.8±78.5
Control	200.5	870.0±65.7
Con-15	150.0	1244.0±252.0
Con-30	290.0	1249.0±177.0

[1] Result are expressed as the units of anti rotavirus activity extrapolated from the standard curve of the standard serum (See chapter-2.8.2).

* Indicate the mean of pooled serum of 5 animals in each group.

**Indicate the mean±SEM of 5 animals in each group.

intravenously compared with controls. Cooper et al (1974) have shown that mice fed a chronic protein deficient diet (8 % protein diet) demonstrated no significant depression of either primary or secondary antibody responses to Brucella abortus antigen when immunized intramuscularly, but a significant reduction of haemagglutinin antibody titres were observed in response to SRBC immunized by intravenous injection as compared to controls. These authors have suggested that the level of humoral immunity observed depends on the nature of the antigen used as stimulus. Cooper et al (1974) used restricted protein diet rather than total food intake restriction since they fed an iso-caloric diet to the control mice. In malnourished children the antibody levels against tetanus toxoid appeared similar to those of control healthy children following intramuscular immunization (Chandra, 1972). Reddy et al (1976) have shown that in response to diphtheria and tetanus toxoid the antibody levels in children with severe protein-calorie malnutrition (PCM) was normal when compared to the normal healthy children. However, this group have shown that the antibody response to typhoid antigen was impaired in children with severe PCM (body weight less than 60 % of normal) but not in mild to moderately (60 to 70 % of standard body weight) malnourished children. These studies indicate that the humoral immunity is affected in severe PCM.

8.3.7. Cell mediated immunity:

In our vitamin A experiment, the pairfed animals had a normal delayed hypersensitivity response to the skin contact antigen indicating normal cell mediated immunity in low food intake. Although the pairfed animals had eaten only about 10-12 % less food than the control ad libitum group. We decided to investigate the relationship between cell-mediated immunity and the level of food restriction. The delayed-type of hypersensitivity response (as expressed by the percentage in increase in ear thickness) of all dietary groups are shown in figure: 8.5. There was a significant increase in the ear

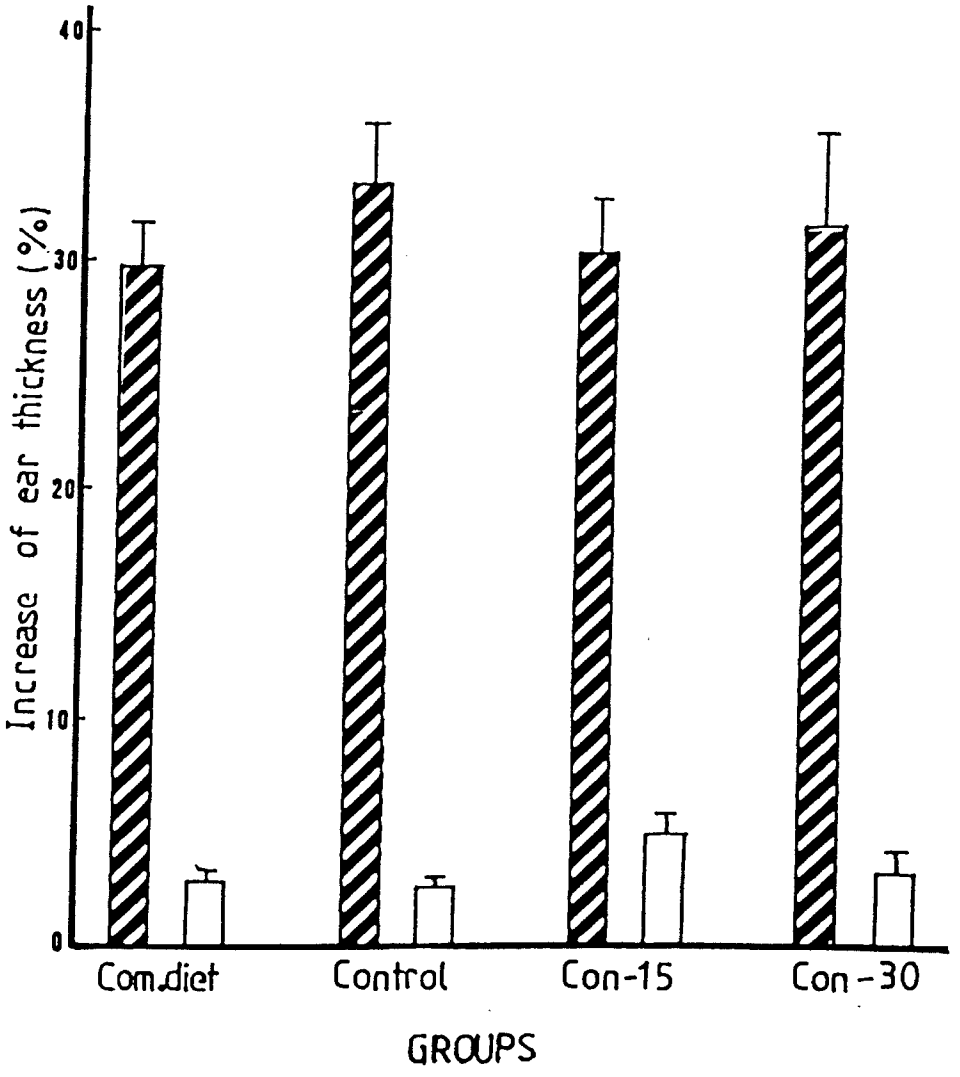

Figure: 8.5 A histogram showing the delayed type hypersensitivity responses of different groups in moderate food restriction to the skin contact allergen picryl chloride. Infected (▨) and non-infected (□).

thickness of immunized animals when compared with non-immunized controls irrespective of any dietary treatment ($P<0.01$). There was no significant change in the increase of the ear thickness observed between any of the dietary groups. This result indicates that reduced food intake does not impair cell mediated immunity (CMI), when delayed hepersensitivity response is used as an index of CMI. Fernandes et al (1976) have shown that mice restricted in calories (37.5 % less intake than normal intake) displayed an increased cell-mediated response to the mitogen phytohemagglutinin and concanavalin A as compared to controls. These authors have also shown a normal B-cell response to the mitogen lipopolysaccharide (Escherichia coli) as compared to controls. Good et al (1976) have shown that in mice chronically fed a protein deficient but iso-caloric diet, the cell mediated immunity was enhanced as judged by the Graft versus host (GvH) reactivity of spleen cells and skin allograft rejection. This group have also demonstrated a very marked enhancement of the proliferative response of spleen cells of chronic protein deficient mice to stimulation by phytohemagglutinin. In vitro cellular cytotoxic responses following tumor immunization (mastocytoma ascites tumor) were normal in mice fed at least 5 % protein diet when compared to control mice (Jose and Good, 1973). These authors have also shown that when mice on a low protein diet are given 50 % fewer calories the cytotoxic lymphocyte function was not impaired. In contrast, Chandra (1972) has reported a significantly lower delayed hypersensitivity response to dinitrochlorobenzene in malnourished children when compared with normal healthy controls. Reddy et al (1976) have shown unaltered cell mediated immunity in children with milder grades of malnutrition (body weights ranges from 71 to 80 % of normal healthy children) as ^{3}H-thymidine incorporation was normal in response to phytohemagglutinin.

8.4. Conclusions :

The present study was designed to investigate the effect of

reduced food intake on rotavirus infection and immunity. This study has shown that reduced food intake is associated with impaired growth in general an observation which is in agreement with others (Brown and Guthrie, 1968; Badger et al., 1972; Cooper et al., 1974). As the degree of food restriction increased the impairment of growth was higher. The spleen and thymus weights were unaffected by feeding low food for 7 weeks after weaning. Rotavirus infection and picryl chloride immunization resulted in a significant increase in the spleen weights and a decrease in the thymus weights of all dietary groups as compared to non-challenged groups. However, it was not possible to explain these changes in the lymphoid organs by histological examination. Others have shown that rats fed a protein deficient diet for 5 weeks had spleen weight about 60 % of controls and have suggested that the loss of splenic tissue resulted from the reduction in cell numbers (Kenney et al,. 1968). McAnulty and Dickerson (1973) fed weanling rats a highly restricted amount of food for four weeks and produced significantly low thymus weights with low DNA per gram of fresh thymus tissue, indicating a reduction in cell numbers.

It is evident from this study that mice on an intake up to 30 % less than the controls for 7 weeks did not have any histopathological changes in the gut after rotavirus challenge indicating normal resistance to rotavirus infection. Fernandes et al (1976) have investigated the influence of calorie restriction (37.5 % less than normal intake) on the development of spontaneous mammary adenocarcinoma in female mice. Calorie restriction had no effect on the life span and completely prevented development of spontaneous mammary tumor in females, whereas control females remained susceptible to the development of mammary adenocarcinoma.

The antibody response following oral challenge of rotavirus was found to be normal in the mice fed reduced food (up to 30 % less). Indicating that a reduction in food intake for 7 weeks does not alter the normal humoral immune function. The normal response to rotavirus infection in food restricted animals indicates that the changes observed in vitamin A deficient animals were a vitamin specific

effect.

Delayed type hypersensitivity, as an index of cell mediated immunity, appeared normal in mice fed up to 30 % less diet than control for the 7 week period. In malnourished children, both the humoral and cellular immunity appeared either normal or impaired depending upon the degree of malnutrition and the antigens used (Chandra, 1972; Reddy et al., 1976). In a review, Chandra (1979) has shown that delayed hypersensitivity and T cell number were lower in malnourished children but that serum immunoglobulins and antibody response appeared normal. On the other hand, laboratory investigations of experimental protein-calorie malnutrition or moderate to severe protein deficiency demonstrated impaired circulating antibody production (Kenney et al., 1968; Cooper et al., 1974). In contrast, cell mediated immunity in mice fed a diet restricted in protein or protein and calories was not diminished , and in some instances was even increased (Jose and Good, 1973; Cooper et al., 1974; Good et al., 1976).

In man it is not always possible to observe pure protein-calorie malnutrition in children, since PCM is usually associated with deficiencies of other micronutrients. Thus the effect of PCM will vary in man depending upon these complicating factors. In experimental animals, the effect of protein or protein-calorie deficiency on the immune response can be standardised. Several studies have shown that cell mediated immunity is either normal or increased (Jose and Good,1973; Cooper et al., 1974; Good et al., 1976) which appears to be in agreement with our findings. On the other hand, humoral immunity or antibody production in protein-calorie deficient animals were found to be reduced which is contrary to our findings. The reason for this discrepancy could be due to the route of immunization. Others have used the intramuscular or intravenous route for immunization but in the present study rotavirus was given orally. Another important point may be the degree of food restriction and also the period on the restricted diet. It is possible that the period of food restriction may not have been sufficient to demonstrate any

significant change in antibody production. Therefore we continued to investigate the effect of food restriction for a longer period and also more restricted diet on antibody production. In order to clarify the effect of the route of immunization we also immunized animals by both the oral and intramuscular routes.

CHAPTER NINE

EFFECT OF SEVERE FOOD RESTRICTION ON THE IMMUNE RESPONSE AGAINST ROTAVIRUS

CHAPTER NINE

9.1. Introduction:

Metcoff et al (1948) have demonstrated that protein-calorie malnourished rats show no significant difference in the incidence of bacteraemia when compared with controls following intraperitoneal injection of virulent S.typhimurium. They have also demonstrated no significant impairment of the circulating antibody response following severe protracted protein deficiency throughout 28 days of the postinfection period. Further, the antibody titres of protein-calorie deficient rats following reimmunization with S.typhimurium O antigen were if anything slightly higher than those of controls. Good et al (1976) have shown that mice on an 8 % protein diet had an increased resistence to experimental infection from pseudorabies virus over a wide range of doses but had a lowered resistance to Group A type 6 Streptococci as compared to controls fed a high protein diet. The same group have also shown that antibody levels in response to Brucella abortus in protein deficient mice were similar to those of controls. The antibody levels in response to SRBC however were lower in the protein deficient mice. Wissler (1947) has shown that immunized, protein deficient adult Albino rats had a marked depression of resistance to experimental pneumococcal infection as compared to well fed controls as judged by their survival rates. Rats fed a control diet limited to the quantity eaten by the low protein fed animals had 100 % survival rates following pneumococcal infection, a survival rate which was similar to the controls whereas survival rates of less than 50 % were seen in protein-calorie deficient rats (Wissler, 1947). Kenney et al (1968) have shown that when adult male rats are fed a low protein diet for 5 weeks they show significantly lower circulating antibody levels as compared to controls following intravenous immunization with SRBC. Offor et al (1985) have demonstrated the effect of malnutrition on the rotavirus replication patterns and

severity of clinical disease in suckling mice. They have shown that the viral replication in the dispersed enterocytes occured earlier and fecal viral sedding peaked significantly earlier than in controls. Further the clinical diarrhoea appeared to be more severe in the malnourished mice, evidenced by the appearence of fecal staining of rotavirus.

Studies prior to 1967, as reviewed by Scrimshaw et al (1968) suggested that although total serum immunoglobulins are within the normal range or are increased in malnutrition, great variability in the levels of individual immunoglobulin classes can occur. In a recent review, Gross and Newberne (1980) have also suggested that immunoglobulin levels are either normal or increased in protein-calorie malnourished children. Chandra (1972) has demonstrated that the antibody levels in malnourished children following tetanus toxoid immunization were similar to those of normal healthy children. However the antibody response following immunization with S.typhi was found to be lower in the malnourished child. Reddy et al (1976) have also shown that protein-calorie malnourished children had significantly lower antibody levels in response to typhoid antigen while the response to tetanus and diphtheria toxoid was normal in all cases. Chandra (1979) has reviewed the information relating to antibody responses in malnourished individuals and shown that in general the response to most antigens is adequate with few exceptions, e.g. heterologous red blood cells (Chandra, 1975) and Salmonella typhi (Chandra, 1972).

Previously we have demonstrated that a reduction in food intake of up to 30 % for a period of 7 weeks, does not impair the resistance to rotavirus infection in mice, judged by the histopathology of the gut and also leaves specific rotavirus antibody levels unimpaired (chapter-eight).

Nalder et al (1972), however, have shown that weanling rats fed a diet for 6 weeks with only a 10% reduction in the nutritional quality relative to the control diet by substitution with sucrose, had a 50% decrease in antibody titres compared with controls following

immunization with S.pullorum antigen. With additional reduction in nutritional quality of the diet the antibody titres were correspondingly decreased. They have suggested that antibody production is sensitive to small differences in dietary quality.

The present study was designed to investigate the effect of severe undernutrition on rotavirus infection by giving a severely restricted food intake for a long period. The antibody responses were investigated, using a different route of presentation.

9.2. Experimental procedure:

The study protocol is presented in figure :9.1. The routine procedures are already described in chapter-2.

9.2.1. Procedure for immunization :

Male Porton mice, 28 days old, were weight matched and divided into three groups. Group-1 (Control) was fed control diet ad libitum, as shown in chapter-2.2. Group-2 (Con-30) and group-3 (Con-50) were fed 30 and 50% less control diet respectively, than the group-1 animals had eaten on the previous day. The animals were housed in groups of three in plastic cages. The room temperature was 20±2°C, humidity 50±5 % and 12 hours lighting schedule per day. The body weight of the animals was taken weekly and food consumption measured daily.

All animals were kept on their respective diet for 11 weeks and each group was divided into three subgroups. Animals from one subgroup were infected with 30 µl/mouse of EDIM rotavirus by oral dosing and termed "infected". Animals from the second subgroup were immunized with 30 µl/mouse of EDIM rotavirus by intramuscular immunization and termed "immunized". Animals from the third subgroup were dosed with phosphate buffer saline (30 µl/mouse) both intramuscularly and orally and termed "non-infected".

One week post-dosing or immunization, the animals were bled

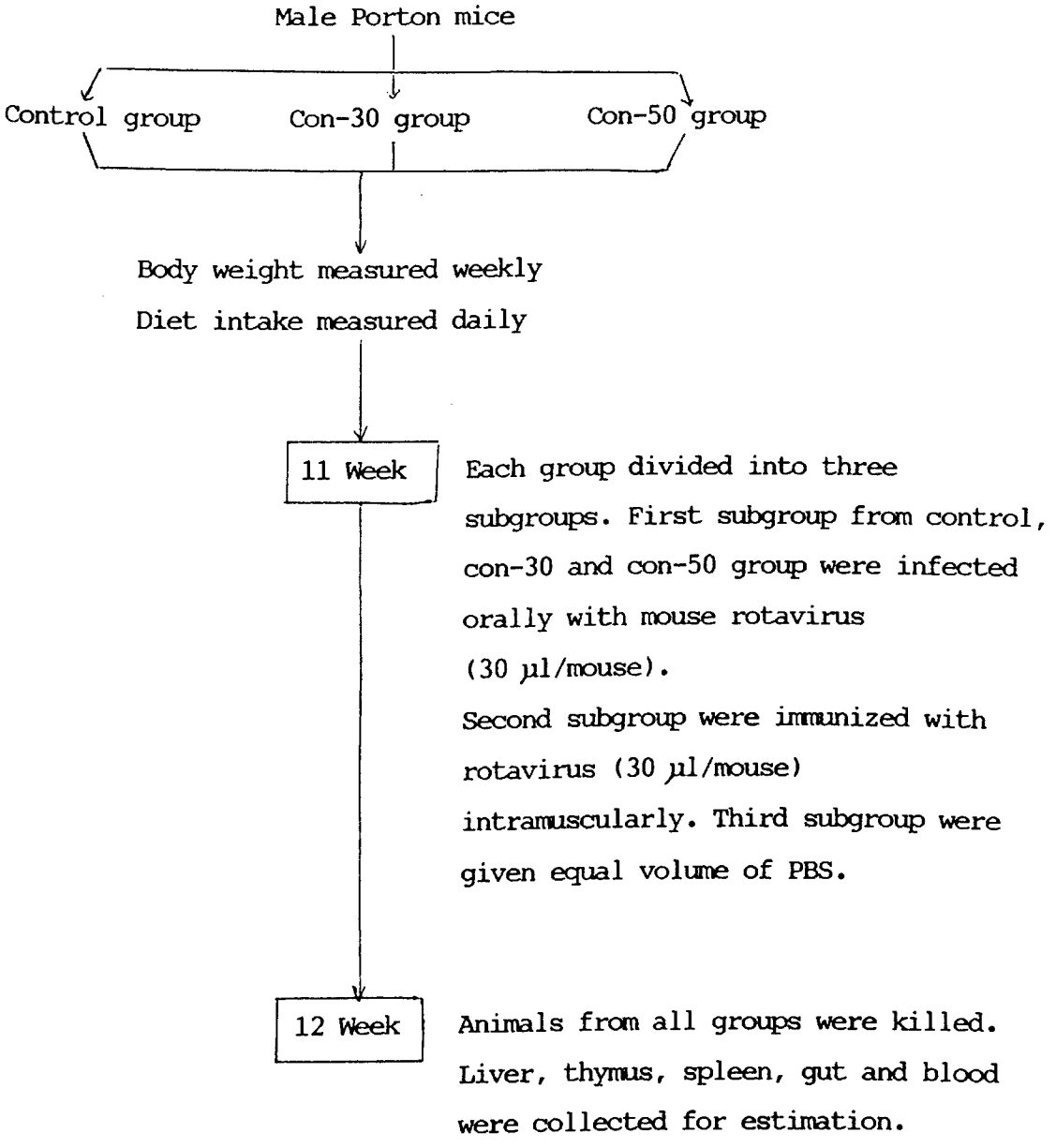

Figure:9.1 Scheme of the procedure for the study of the effect of severe food restriction on the immune response to rotavirus.

by heart puncture and blood samples were allowed to stand at room temperature for one hour to allow clot formation. After clotting, the blood was centrifuged and the clear serum removed with a pasteur pipette. The serum samples were stored in ependorff tubes at -20°C until analysed. Immediately after the blood was collected, the carcase of the animals was cut open and the liver, spleen and thymus excised and weighed. Liver was then stored at -20°C until analysed for vitamin A content. Spleen, thymus and the middle portion of small intestine were fixed in 10 % formalin buffered solution for histological analysis.

9.2.2. Determination of vitamin A:

9.2.2.1. Liver:

Liver vitamin A was determined by the method of Bayfield (1975) with the modifications (Section-2.3).

9.2.2.2. Serum:

Serum vitamin A was estimated by the high performance liquid chromatography (HPLC) method of Bieri et al (1979) with slight modification (section-2.4.2).

9.2.3. Histological study:

The histology of the spleen, thymus, and gut was examined by the method described in section-2.5.

9.2.4. Antibody assay:

Total circulating antibody specific to rotavirus antigen was measured using the ELISA method described in section-2.8.2.

9.2.5. Statistical analysis:

Results are expressed as the mean and standard error of the mean. Differences between groups were evaluated by using 2-way analysis of variance (ANOVA) for independent means for the parametric data and Wilcoxon rank sum test for the non-parametric data.

Differences of the means were considered significant when $P<0.05$.

9.3. Results and discussions:

9.3.1. Body weights:

The body weights of all groups of animals up to 11 weeks on their respective diets are shown in figure-9.2. The animals in the con-30 and con-50 groups were fed 30 and 50% less food than the control group and gained much less weight than the controls. Animals in the con-50 group gained even less weight than the con-30 group. 11 weeks after feeding, animals from each group were divided into three subgroups and infected with rotavirus as described in the experimental procedure (9.2.1). The average body weights of all groups of animals at 11 (time of infection) and 12 weeks (time of killing) are shown in table: 9.1. After 11 weeks of feeding, the body weights of con-30 and con-50 were on average 67 and 46% of the control animals respectively. At this time, the body weights of con-30 and con-50 groups were significantly lower than that of control group irrespective of rotavirus challenge ($P<0.01$, univariate level). The body weights of the con-50 group were significantly lower than the con-30 group irrespective of infection ($P<0.01$, univariate levels).

At 12 weeks (one week after the rotavirus challenge), the body weights of con-30 and con-50 were significantly lower than those of the control group irrespective of rotavirus challenge ($P<0.01$, univariate level). Further, the body weights of the con-50 group were significantly lower than the con-30 group irrespective of infection

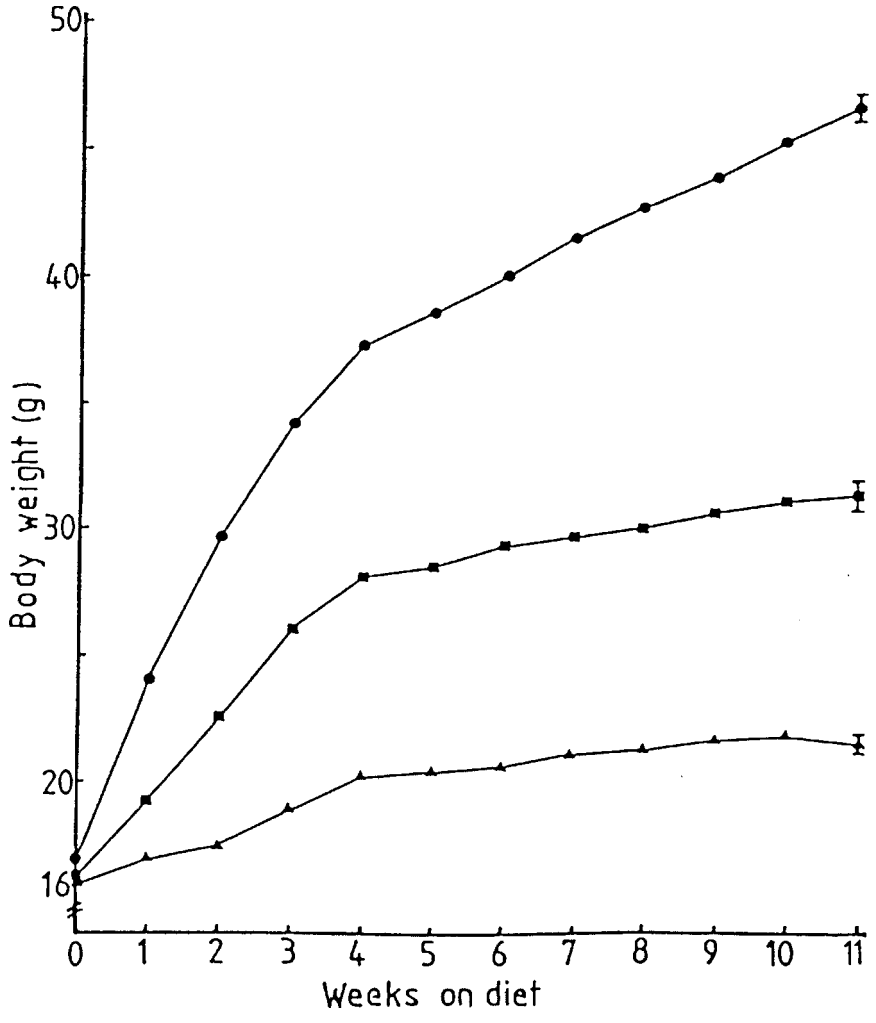

Figure: 9.2 Body weight gain of different groups of animals during severe food restriction. Control (●—●), con-30 (■—■) and con-50 (▲—▲) groups. Each point represents the mean of 18 animals. Mean±SEM at 11 weeks.

Table: 9.1

Change in body weight due to rotavirus challenge in the severe food restriction [1]

Group	Time Week	Non-infected g	Infected g	Immunized g
Control	11	46.9±1.0	46.6±1.6	46.8±1.0
	12	47.8±0.9	46.8±1.5	47.1±1.0
Con-30*	11	31.5±1.0	31.3±0.9	31.5±1.0
	12	31.3±1.0	30.7±0.9	30.8±1.1
Con-50**	11	22.0±0.6	21.3±0.6	21.5±0.7
	12	21.7±0.5	21.1±0.6	21.5±0.7

[1]Results are expressed as a mean±SEM of 6 animals in each group.

*Significantly different from control group irrespective of infection at 11 & 12 week time point.

**Significantly different from the control and con-30 groups irrespective of infection at 11 & 12 time point.

($P<0.01$, univariate levels). There was no significant change in body weight due to the rotavirus challenge. In combination with diet rotavirus challenge did not have any significant influence on the body weight gain. In other words there was no significant interaction of diet and infection. On average the final body weights of the con-30 and con-50 groups were 34.5 and 54.5% less than those of the control ad libitum group irrespective of infection. Nalder et al (1972) have shown that weanling rats fed a diet with only a 10 % reduction in the nutritional quality of the control diet, by sucrose dilution, had a significantly reduced body weight compared to controls. With an additional decrease in the nutritional value of the diet the body weight was further decreased. For example, a 30 or 50% reduction in the quality of the diet gave body weights which were 33 and 58% less than the control values. Mittal and Woodward (1985) have shown that weanling mice fed 50% of the average ad libitum intake (g feed/g body weight/day) for 14 days had a high mortality rate (about 60%). However, this group have also shown that mice fed 60% of the average ad libitum intake (body weight basis) for 14 days resulted in a 30% loss of initial body weights and a moderate mortality rate (about 19%). This result differs from our findings. Mittal and Woodward (1985) have demonstrated the effect of severe food restriction by giving diet on the body weight basis. In the present study the animals were fed a reduced diet on the basis of the average of food consumption of the controls and not on a body weight basis.

9.3.2. Organ weights:

9.3.2.1. Liver weight:

The liver weights of non-infected, infected and immunized groups are shown in table: 9.2. There was no significant difference in total liver weight due to rotavirus infection or immunization irrespective of dietary treatment. Further, no significant interaction was observed between the diet and rotavirus challenge. However, there

Table: 9.2

Effect of severe food restriction and rotavirus challenge on the liver weights.[1]

	Total weight			As % of body weight		
Group	Non-infected g	Infected g	Immunized g	Non-infeced %	Infected %	Immunized %
Control	1.82±0.04	1.71±0.1	1.71±0.07	3.82±0.1	3.65±0.2	3.62±0.1
Con-30*	1.05±0.04	1.10±0.03	1.07±0.04	3.35±0.05	3.59±0.1	3.50±0.04
Con-50**+	0.73±0.02	0.72±0.01	0.78±0.04	3.37±0.1	3.4±0.06	3.65±0.05

[1]Results are expressed as the mean±SEM of 6 animals in each group.

*Significantly different from the control group ($P<0.01$) irrespective of rotavirus challenge when compared for the total weight.

**Significantly different from the control and con-30 groups ($P<0.01$) irrespective of rotavirus challenge when compared for the total weight.

+ Significantly different from the control group ($P<0.05$) only irrespective of rotavirus challenge when compared for the relative weight.

was a significant difference observed between different dietary groups irrespective of rotavirus challenge (univariate levels). Total liver weights of the con-30 and con-50 groups were significantly lower than the control group irrespective of infection ($P<0.01$). The total liver weights of the con-50 group were significantly lower than the con-30 group irrespective of infection ($P<0.01$).

The liver weights calculated as a percentage of body weight for the con-50 group were significantly lower than those of the control group irrespective of infection ($P<0.01$, univariate level). There was no significant difference observed in the relative liver weights of the con-30 group in comparison with either the control or con-50 groups irrespective of infection. No significant difference was observed however due to the rotavirus infection or immunization. Further, there was no significant interaction observed between the diet and infection.

9.3.2.2. Spleen weight:

The spleen weights of non-infected, infected and immunized animals are shown in table: 9.3. There was no significant difference in the total spleen weights observed due either to rotavirus infection or immunization irrespective of dietary treatment. No significant interaction was observed between diet and infection. The total spleen weights of con-30 and con-50 were significantly lower than the control group irrespective of infection ($P<0.01$, univariate level). The spleen weights of con-50 were significantly lower than those of con-30 irrespective of infection ($P<0.01$, univariate level).

There was no significant change observed in the relative spleen weight (as % of the body weight) due to rotavirus infection or immunization irrespective of dietary treatment. Also there was no interaction observed between diet and infection. The relative spleen weights of con-30 group appeared significantly higher than either control or con-50 groups irrespective of infection ($P<0.01$, univariate level). However the relative spleen weights of con-50 were similar to

Table: 9.3

Effect of severe food restriction and rotavirus challenge on the spleen weights.[1]

	Total weight			As % of body weight		
Group	Non-infected mg	Infected mg	Immunized mg	Non-infected %	Infected %	Immunized %
Control	86.3±2.3	83.2±2.8	98.3±5.7	0.18±0.01	0.18±0.01	0.21±0.01
Con-30*+	70.0±3.0	77.3±4.0	73.1±7.0	0.23±0.01	0.25±0.01	0.24±0.02
Con-50**	42.3±1.9	44.5±3.0	40.1±2.3	0.20±0.01	0.21±0.01	0.19±0.01

[1]Results are expressed as the mean±SEM of 6 animals in each group.

*Significantly different from the control group ($P<0.01$) irrespective of rotavirus challenge when compared for the total weight.

**Significantly different from the control and con-30 groups ($P<0.01$) irrespective of rotavirus challenge when compared for the total weight.

+ Significantly different from the control and Con-50 group ($P<0.01$) irrespective of rotavirus challenge when compared for the relative weight.

those of the control groups irrespective of infection. The results indicate that when animals were fed 50% less food than controls, their spleen weights were affected in proportion to their body weight. On the other hand, when animals were fed 30% less food than controls, their spleen weight was affected at a slower rate than their body weight gain. Mittal and Woodward (1985) have shown that weanling mice fed 60% of the average ad libitum intake (body weight basis) for 14 days had a splenic index (mg/g body weight) of about 33% of control well fed animals indicating a severe reduction in the total and relative spleen weights as compared to that of controls.

Finally, both the total and relative spleen weights of control animals following intramuscular immunization appeared greater than the other control groups. In the case of con-30 and con-50 there was no such difference observed. However, we could not demonstrate that this difference was statistically significant using two way analysis of variance.

9.3.2.3. Thymus weight:

The thymus weights of infected , non-infected and immunized groups are shown in table: 9.4. There was no significant difference observed in the total thymus weight due to rotavirus infection irrespective of dietary treatment (univariate level). In combination with diet rotavirus infection or immunization did not have any significant influence on the total thymus weight. The total thymus weights of the con-30 and con-50 groups were significantly lower than the control group irrespective of infection ($P<0.01$, univariate level). Further, the total thymus weights of the con-50 group were significantly lower than those of the con-30 group irrespective of infection ($P<0.01$, univariate level).

Rotavirus infection or immunization had no effect on the relative thymus weight irrespective of any dietary treatment (univariate level). There was also no interaction between the administered diet and infection. The relative thymus weights of the

Table: 9.4

Effect of severe food restriction and rotavirus challenge on the thymus weights.[1]

	Total weight			As % of body weight		
Group	Non-infected mg	Infected mg	Immunized mg	Non-infected %	Infected %	Immunized %
Control	53.0±3.3	51.8±2.6	50.7±2.6	0.11±0.01	0.11±0.01	0.11±0.01
Con-30*	40.3±2.9	39.5±2.9	39.3±3.1	0.13±0.01	0.13±0.01	0.13±0.01
Con-50**+	30.3±2.0	26.9±3.5	31.3±2.3	0.14±0.01	0.13±0.02	0.15±0.01

[1]Results are expressed as the mean±SEM of 6 animals in each group.

*Significantly different from the Control group ($P<0.01$) irrespective of rotavirus challenge when compared for the total weight.

**Significantly different from the control and con-30 groups ($P<0.01$) irrespective of rotavirus challange when compared for the total weight.

+ Significantly different from the control group ($P<0.01$) irrespective of rotavirus challange when compared for the relative weight.

con-50 group were significantly higher than the control group irrespective of rotavirus challenge ($P<0.01$, univariate level). Although the relative thymus weight of the con-30 group appeared higher than controls, the difference was not significant. Higher relative thymus weight in the con-50 group indicates that body weight was affected more than thymus weight. In fact the total thymus. weight of the con-50 group was about 43% less than in controls irrespective of infection. The body weights of con-50 animals were about 55% less than control values irrespective of infection. Brown and Guthrie (1968) have demonstrated that severely undernourished rats had a significantly higher relative thymus weight than controls. This group has suggested that the body weight of undernourished (protein-calorie restricted) rats were depressed to a greater extent than all other organ weights. Mittal and Woodward (1985) on the other hand, have shown that mice fed 60% of the average ad libitum intake (body weight basis) had a thymus index of 10% that of controls indicating a severe reduction in the total and relative thymus weight.

9.3.3. Vitamin A levels:

9.3.3.1. Liver vitamin A:

The liver vitamin A content of non-infected, infected and immunized animals are shown in table: 9.5. Rotavirus infection or immunization had no effect on the liver vitamin A reserve irrespective of dietary treatment (univariate level). Rotavirus infection or immunization did not have any significant influence on the liver vitamin A reserve in combination with diet. The liver vitamin A reserve of the con-50 group was significantly lower than control and con-30 groups irrespective of infection ($P<0.01$, univariate level). The liver vitamin A reserve of con-30 appeared similar to control values irrespective of infection. These results indicate that a reduction in food intake by up to 30 % does not affect the liver vitamin A reserve and is in agreement with our previous results

(chapter-8). Although the con-50 animals had eaten 50 % less diet and thus 50 % less vitamin A, their liver vitamin A reserve appeared only 30 % less than that of controls. Our results indicate that the vitamin A utilization is dependent on the growth of the animals as suggested by Rechcigl et al (1962). Zaklama et al (1972) have also shown that rats on a low protein diet eat about 30 % less food over an 8 weeks period and have similar vitamin A reserves as control rats fed a high protein diet. These authors have suggested a higher rate of utilization of vitamin A in rats on a high protein diet.

9.3.3.2. Serum vitamin A:

The serum vitamin A levels of all groups are shown in table: 9.6. There was no significant difference in serum vitamin A levels due either to rotavirus infection or immunization irrespective of any dietary treatment. Further there were no significant differences in serum vitamin A levels in any of the dietary groups irrespective of infection. No significant interaction was observed between diet and infection. Zaklama et al (1972) have shown that protein-calorie malnourished rats have similar vitamin A levels to control rats fed a high protein diet and support our finding. Underwood et al (1979) have shown that when the liver reserve of vitamin A is above 5 µg/liver wet weight it is capable of maintaining normal plasma retinol levels.

9.3.4. Histological study :

9.3.4.1. Spleen:

The histology of the spleen in all groups was examined by light microscopy. The B and T cells areas of con-30 and con-50 animals appeared similar to the control ad libitum group. Further it was not possible to demonstrate any significant change in the B or T cell areas in either non-infected, infected or immunized animals. The

Table: 9.5

Effect of severe food restriction and rotavirus challenge on the liver vitamin A[1]

Group	Non-infected µg/liver	Infected µg/liver	Immunized µg/liver
Control	245.2±13.4	250.7±10.9	243.3±12.7
Con-30	223.8±11.3	234.9±13.2	240.8±20.7
Con-50*	167.5±6.3	178.0±8.0	165.5±5.8

Table: 9.6

Effect of severe food restriction and rotavirus challenge on the serum vitamin A[1]

Group	Non-infected µg/100ml	Infected µg/100ml	Immunized µg/100ml
Control	48.7±2.4	52.2±3.1	50.6±3.1
Con-30	44.4±2.8	45.3±1.5	45.6±3.0
Con-50	53.5±3.8	46.9±1.5	49.3±2.4

[1]Results are expressed as the mean±SEM of 6 animals in each group.
*Significantly different from the control and con-30 irrespective of rotavirus challenge ($P<0.01$).

histology of all groups appeared normal. Lower total spleen weight reflects smaller size and less cellularity without affecting structure. Kenney et al (1968) have shown that protein deficient rats have a spleen weight of about 60 % of controls and have suggested that the loss of splenic tissue results from a reduction in cell numbers. Mice on a 4 % protein diet for 4 weeks had a significantly lower spleen weight compared with controls and the cell content was directly proportional to the weight of the organ (Bell et al., 1976). This group have also demonstrated histological changes in protein-calorie deficient mice, for example a reduction in the size of lymphoid follicles. However, we could not demonstrate any such histological changes in undernourished mice.

9.3.4.2. Thymus :

The structure of the thymus of both infected and non-infected animals was examined by light microscopy. Both the cortical and medullary areas of the thymus of the con-30 and con-50 groups appeared normal. Although the thymic lobes of the undernourished animals were smaller, it was not possible to differentiate any obvious histological changes. Mittal and Woodward (1985) have reported that mice fed 60% of the average ad libitum intake (on body weight basis) exhibited cortical lymphocyte depletion, histologically.

9.3.4.3. Gut :

The histology of both the non-infected and infected gut (small intestine) was examined by light microscopy. The histology of the gut of infected and non-infected controls appeared normal. Both groups had intact villi. The gut histology of the non-infected con-30 animals was similar to infected con-30 animals and showed intact villus tips. There was no significant difference observed due to rotavirus challenge.

The animals fed 50% less food than normal intake (con-50) for

12 weeks showed a severe destruction of the villus tips in both non-infected and infected individuals. The lamina propria of the villus was exposed to the lumen of the gut. However, the degree of the damage to the villus tips in the infected con-50 animals was not obviously more than the non-infected animals. There was a significant reduction in the length of the villi of con-50 animals as compared to control or con-30 irrespective of infection. It was not therefore possible to differentiate the specific effects of rotavirus infection in these animals. Hence the damage to the gut of the con-50 animals was mostly due to the extreme reduction in food intake rather than rotavirus infection. Representative photographs of groups of both non-infected and infected animals are shown in figure: 9.3.

9.3.5. Serum antibody levels specific to rotavirus:

Total serum antibody specific for rotavirus following oral challenge was measured for all dietary groups and the results are presented in table: 9.7. The serum antibody levels specific to rotavirus in the non-infected groups served as the background antibody levels in these animals. 7 days after the oral challenge with rotavirus there was a significant increase in the antibody response compared with non-infected animals irrespective of dietary treatment. There was no significant difference in the serum antibody levels observed among different dietary groups. The results indicate that in mice, a reduction in dietary intake of either 30 or 50% less than the control value does not affect humoral immunity following oral challenge of rotavirus. In malnourished children total serum immunoglobulins were found to lie within the normal range or to be increased, but increased levels of serum IgA are frequently seen (Scrimshaw et al.,1968). On the other hand, McMurray et al (1976) have shown that the secretory IgA (SIgA) levels in tears and saliva of both moderately and severely malnourished children were significantly depressed.

Total serum antibody levels specific to rotavirus following

Figure: 9.3. Representative photographs of the gut of (A) Non-infected control,(B) Infected control, (C) Non-infected con-30,(D) Infected con-30, (E) Non-infected con-50 and (F) Infected con-50 mice . H & E stain, X 112.

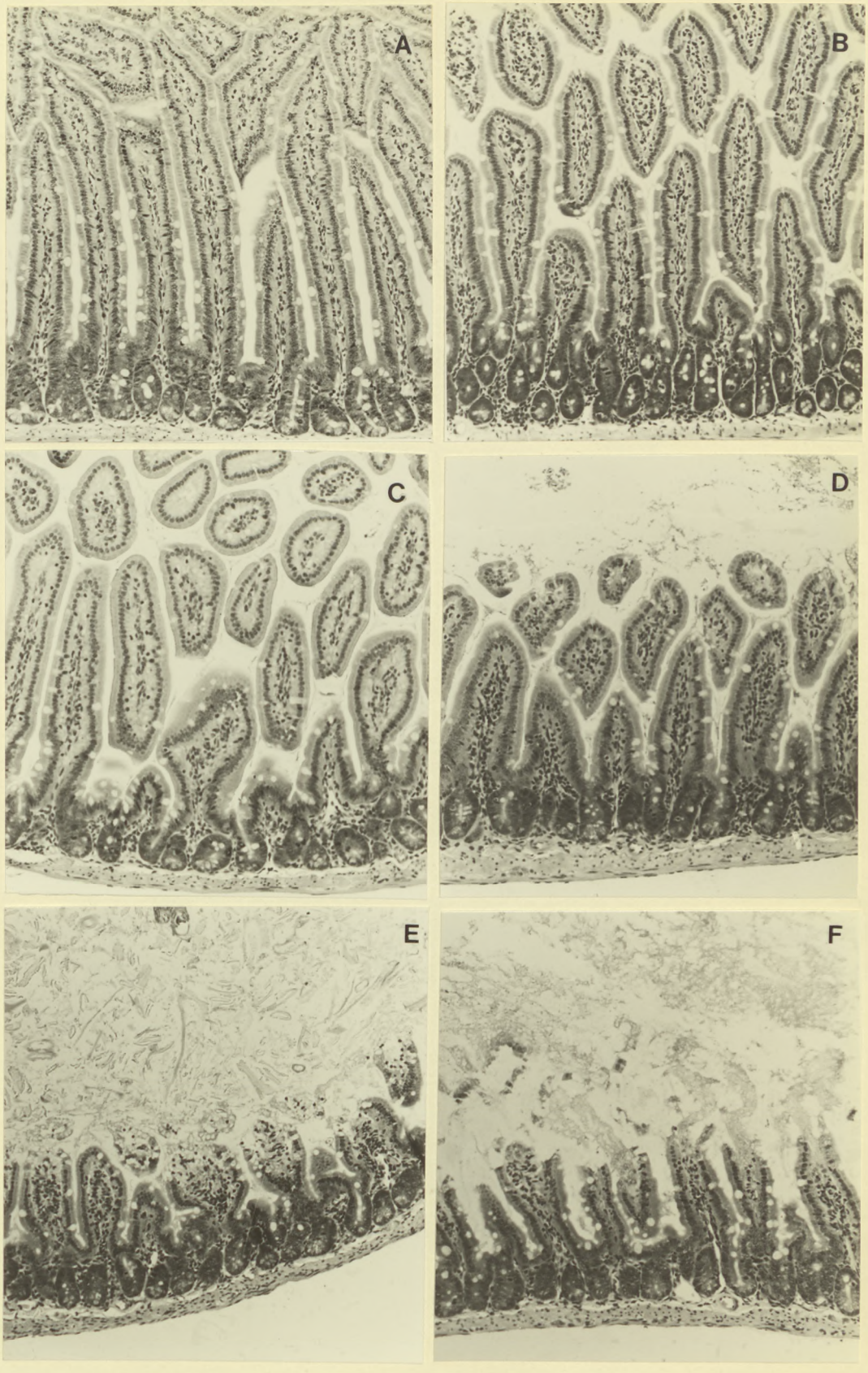
A
B
C
D
E
F

Table: 9.7

Effect of severe food restriction on the serum antibody levels following oral challenge of rotavirus[1]

Group	Non-Infected*	Infected**
	Units	Units
Control	205.0	3041.0±328.0
Con-30	219.0	3481.0±486.0
Con-50	264.0	2906.0±238.0

[1]Results are expressed as the units of anti rotavirus activity extrapolated from the standard curve of the standard serum (see chapter-2.8.2).

*Shows the mean antibody levels of the pooled serum of 6 animals in each group.**Shows the mean±SEM of 6 samples in each group.

intramuscular immunization were also measured for all dietary groups and the results are shown in table: 9.8. The serum antibody levels following intramuscular immunization of con-50 group were significantly lower than that of control ad libitum group. The mean antibody levels specific to rotavirus in the con-50 animals appeared lower than the con-30 animals, however, the difference was not statistically significant. The average serum antibody levels specific to rotavirus of con-30 group was less than half the control animals, but the difference was not statistically significant. The results show a wide variation in the antibody response to rotavirus immunization within each of the dietary groups, figure : 9.4. Because of this wide variation in antibody response, there was no significant difference between control and con-30 groups. The variation in the antibody levels could be due to the use of outbred animals. In outbred animals, due to genetic variation the antibody levels may vary from animal to animal even though they are kept under the same condition.

The mean antibody levels specific to rotavirus were progressively less with increased dietary restriction and differed significantly between con-50 and controls. Nalder et al (1972) have shown that the antibody levels in response to S.pullorum antigen decreased proportionately with the decrease in the nutritional quality of the diet. Jose and Good (1973) have investigated the effect of a progressive reduction in dietary protein or protein-calories on humoral immunity in mice. A significant reduction in serum cytotoxic and haemagglutinin antibody titres were noted at dietary levels of 8% protein as compared to controls following allogenic tumor cell immunization. Further, a slight reduction in antibody levels was noted when calories were reduced to half the normal value on top of protein deficiency. There was a complete disappearence of serum cytotoxic and haemagglutinating activity at a level of 3% protein diet. On the other hand, Metcoff et al (1948) have shown that protein-calorie malnourished rats had no impairment of their circulating antibody response to S.typhimurium antigen. In patients with clinical protein-calorie malnutrition, normal antibody responses to tetanus and

Table: 9.8

Effect of severe food restriction on the serum antibody levels following intramuscular immunization of rotavirus[1]

Group	Immunized* Units
Control	1652.0±461.0
Con-30	727.0±308.0
Con-50**	278.0±35.5

[1]Results are expressed as the units of anti rotavirus activity extrapolated from the standard curve of the standard serum (see chapter-2.8.2).

*Shows the mean±SEM of 6 samples in each group.

**Significantly different from the control ad libitum group by Wilcoxon rank sum test ($P<0.05$).

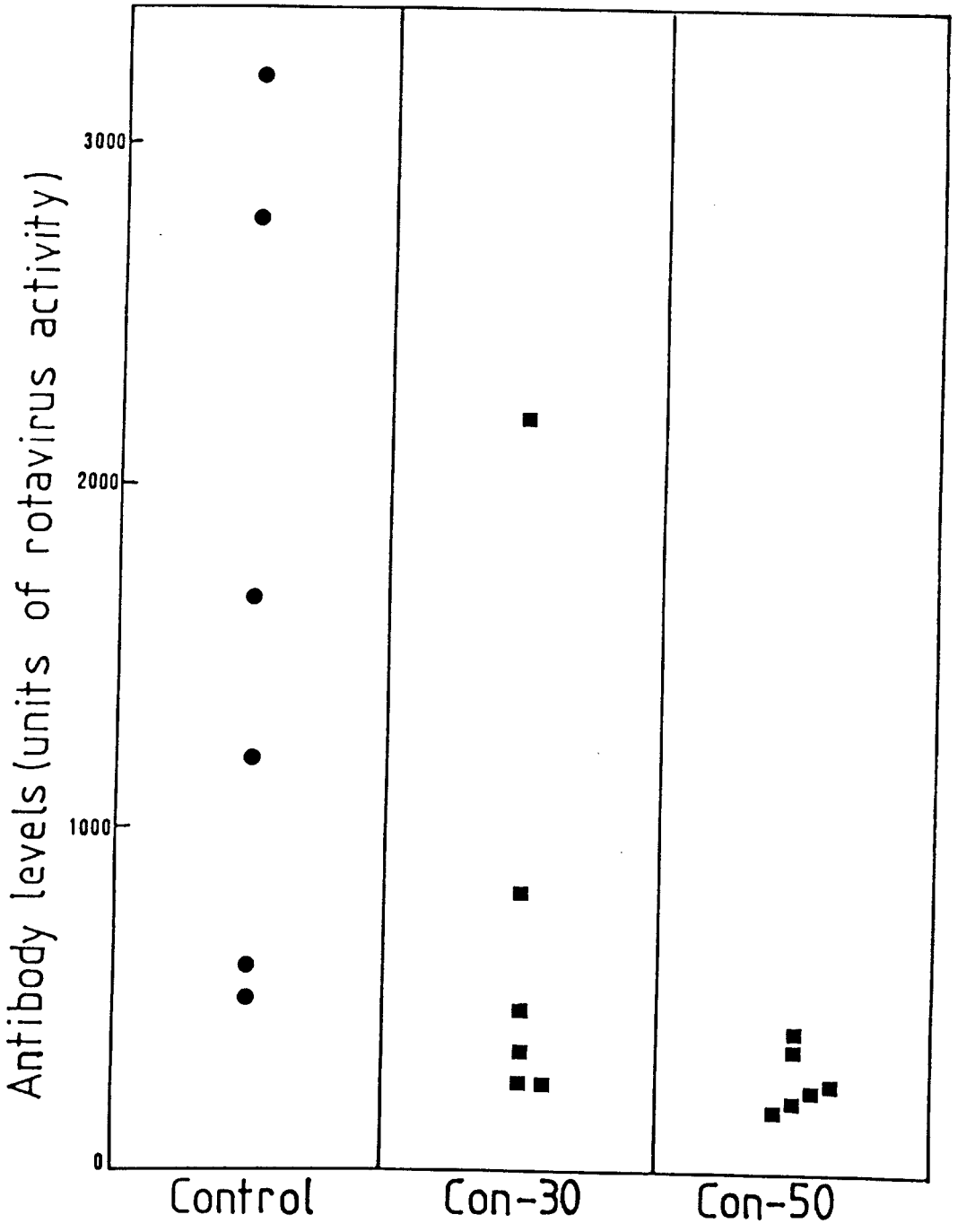

Figure: 9.4 The distribution of circulating antibody levels following intramuscular immunization of different dietary groups in the severe food restriction.

diphtheria toxoid have been reported (Chandra, 1972). In PCM the antibody responses following immunization with viral antigens are variable (reviewed by Gross and Newberne,1980). These studies show that the antibody response in malnourished subjects depends on the type of antigen administered.

Our results have shown that following intramuscular immunization the antibody response to rotavirus is significantly impaired in animals fed 50% less diet than controls. However the responses following oral challenge was normal in animals fed either 30 or 50% less food intake. This result indicates that immune response to oral challenge with rotavirus in undernourished animals is normal. Variation in the antibody response due to the different routes of immunization may reflect the fact that rotavirus in the gut is a proliferating antigen. Following oral challenge the antigen load available to provoke an antibody response is therefore much higher than the original infective dose. Following intramuscular immunization however, the rotavirus antigen does not proliferate and the corresponding antigenic dose is lower and hence the animal's reduced ability to mount an immune response is more obvious.

9.4. Conclusions:

The present study was designed to investigate the effect of severe undernutrition on the antibody response to rotavirus infection. Reduced food intake impaired weight gain compared with controls and as food restriction increased the body weight gain was decreased proportionately. These results are in agreement with our previous findings (chapter-eight) and with the findings of others (Nalder et al., 1972; Bell et al., 1976). In contrast Mittal and Woodward (1985) have shown that when mice were fed 40% less diet than ad libitum controls on a body weight basis they exhibited a 30% loss of initial body weight during 14 days and also a mortality rate of about 19%. Their results differ from our findings. For many years it has been known that diet intake is determined mainly by energy requirement

(Kleiber, 1945) and energy requirement is proportional to the body weight. Thus in the present study, although the animals of the con-30 and con-50 groups had 30 and 50% less calories than controls, they were able to maintain their body weight since the energy requirements were less in these animals.

It is evident from the histological study that the spleen and thymus of undernourished animals were structurally normal, although the total weight of these organs was lower than control, an observation consistant with our previous findings (chapter-eight). The histology of the gut of the animals which experienced a 50% reduction in food intake for 12 weeks showed severe damage to the villus tips irrespective of rotavirus infection, indicating that severe undernutrition itself can lead to damage to the villus tips. It was not possible to isolate the effect of rotavirus infection in addition to dietary restriction in the small intestine. We assume that the rotavirus infection did not cause any additional, obvious damage in undernourished animals which received a balanced (vitamin A balanced) diet but in reduced amount.

The antibody responses following oral challenge were normal in undernourished animals, consistant with our data reported in chapter-eight. This result, indicating that the gut immunity was normal in the undernourished animals, supports our contention that the histopathological changes of the gut in infected vitamin A deficient animals were due to the vitamin A reduction. Following oral challenge there was no change in antibody production in the undernourished mice, providing further evidence that the impaired antibody production in the vitamin A deficient animals is a specific effect of the vitamin A deficiency. However, mice with a reduced food intake have shown a significantly lower antibody level following intramuscular immunization. This result indicates that the antibody response differs depending upon the route of presentation of antigen. Oral challenge would provoke antibody production by stimulating the gut immune system including Peyer's patches, mesenteric lymph nodes and plasma cells in the lamina propria. Whereas intramuscular immunization would stimulate

the systemic immune system, involving spleen and other lymph node chains. Therefore, intramuscular and oral immunization acts through different components of the immune system, this may explain the different responses obtained in the present study. Further we used a replicative antigen and gut is the site of replication. Thus after oral challenge the quantity of antigen available would be much higher than following intramuscular immunization, although the initial dose was the same for both routes. Offor et al (1985) have shown that in malnourished suckling mice a significantly smaller dose of rotavirus could produce active replication than in controls. These authors have suggested that the acceleration in rotavirus replicative events in malnourished animals may be due to a specific alteration in susceptible enterocyte cell membranes as a direct result of nutritional deprivation.

CHAPTER TEN

GENERAL DISCUSSIONS

CHAPTER TEN

General discussions:

The study reported has investigated the effect of vitamin A deficiency on the immune response to rotavirus infection. Vitamin A deficiency is one of the most common deficiencies worldwide (Roels, 1970) and is particularly common in developing countries (Pirie, 1983). Several reports have shown an association between vitamin A deficiency and diarrhoeal disease (Sommer et al, 1984; Stoll et al., 1985; Stanton et al., 1986), however, evidence suggesting that vitamin A deficiency yields an increased risk of diarrhoea is equivocal and further studies are required to fully establish this relationship (Feachem, 1987). Rotavirus, one of the major causes of viral diarrhoea in infants and children throughout the world (WHO Scientific working group, 1980), acts via the epithelium of the intestine (Starkey et al.,1986). Since vitamin A is an essential nutrient for the maintenance of the epithelia of various organs (Olson,1972), we decided to investigate the relationship between vitamin A deficiency and diarrhoeal disease using rotavirus in the experimental animals. To the best of our knowledge this is the first investigation of the interaction of vitamin A deficiency and rotavirus infection.

In the literature most vitamin A deficiency studies have employed rat, hamster or chick models (Muto et al,1972; Krishnan et al.,1976; Nauss et al.,1979; Takagi and Nakano,1983; Nauss et al.,1985; Bonanni et al.,1973; Bang and Foard,1971). Studies employing mice are few (McCarthey and Cerecedo,1952; Smith et al.,1987), and it has been suggested that mice exhibit greater resistance to the generation of vitamin A deficiency defined as body weight change, reduction in life span and the appearance of deficiency symptoms (McCarthey and Cerecedo,1952). Further, mice may differ from rats with respect to vitamin A storage and metabolism (Smith et al.,1987). In the present study a vitamin A deficient mouse model was developed by

feeding an appropriate diet to weanling mice. The mice became vitamin A deficient by 63-70 days of feeding the experimental diet by the criteria of vitamin A levels in serum and liver, and body weight gain (Olson,1988; Wolf,1980). None of the vitamin A deficient animals showed any symptoms of clinical deficiency at any time during the experiment other than a body weight plateau. In the first two experiments (chapter-3 & 5) the serum vitamin A levels were estimated using a spectrophotometric method but in the later experiments more sensitive HPLC methods were used and have confirmed the low levels of vitamin A in the deficient animals. McCarthey and Cerecedo (1952) have shown that in weanling mice vitamin A deficiency symptoms appeared after 90-120 days of feeding. In the present study, studies were completed within 84 days of feeding the experimental diet and demonstrable clinical symptoms of deficiency were not seen. It is possible that the development of clinical symptoms of vitamin A deficiency in an individual requires further complication such as infection. Bieri (1969) has demonstrated that germ free vitamin A deficient rats continued their growth for a longer period and lived longer than conventional vitamin A deficient animals. In fact an important aspect of our study was that the vitamin A deficient animals had no signs of intercurrent infection as judged by the histology of the gut and spleen. Thus, we have been able to demonstrate the effect of rotavirus infection as a primary event in vitamin A deficient mice.

A decrease or loss of appetite occurs in many specific dietary deficiencies including vitamin A deficiency (Kleiber,1945) and therefore a pairfed group was introduced in this study. We were able to demonstrate the effect of pure vitamin A deficiency on rotavirus infection. Although pairfed animals were given the same amount of food as that eaten by the deficient animals, the body weight gain of pairfed animals was significantly higher than vitamin A deficient animals indicating an alteration in metabolic efficiency in the latter. Kleiber (1945) has discussed the observation that a diet deficient in any food constituents tends to result in a decrease in the efficiency of energy utilization, by which he meant the transfer

of food energy into a form of energy useful to individual body. Sampson and Korenchevsky (1932) have suggested that two-fifths of the retardation of growth in vitamin A deficient animals was accounted for by the decrease in food intake or lack of an appetite promoting principle and three-fifths by the lack of an anabolic principle of vitamin A. Takagi and Nakano (1983) have shown that vitamin A deficient rats ceased to grow before their appetite began to fall and their experiment could suggest that vitamin A deficiency leads to decreased efficiency of energy utilization.

Ideally test and pairfed control animals should be kept individually caged. But in the present study the animals were kept in groups of two or three in each cage. During the early experiments we explored the effect of housing animals individually. The animals were fed in groups of 3 for the first 9 weeks and thereafter they were fed individually. Within 2 weeks of individual feeding the animals from control and pairfed groups stopped growing and food intake was decreased. These animals were then rehoused in groups of three per cage and they resumed weight gain. At this point the experiment was discontinued. One of the possible explanations for this behaviour could be the lack of social contact among the animals, but probably most important is the fact that mice are coprophagic. Prevention of coprophagy resulted in a depressed growth rate in rats although these animals were fed a balanced diet (Barnes et al.,1963). These authors have also shown that the growth stimulation is observed only when fecal pellets are ingested directly on extrusion from the anus. Therefore, individual feeding may cause partial prevention of coprophagy from the anus and thus result in growth impairment. However in this study it was not possible to explore this hypothesis in detail and it was judged desirable to house the animals in groups of two or three per cage.

It was observed that the final body weights of different dietary groups in different experiments were variable. Since the animals used were from different batches and obtained at different times variation in their growth was not unexpected. Another point is

the environmental effect of carrying these animals from the Boldrewood animal house, Southampton University, to the Southampton General Hospital animal house. However, within the same experimental batch the body weights of vitamin A deficient animals were always significantly lower than control and pairfed groups. This finding was consistant throughout the study. Further vitamin A supplementation after 10 weeks of feeding deficient diet lead to an increased growth rate (chapter-6) indicating that vitamin A deficiency impairs growth. The final body weight of refed and control animals were however still significantly different, reflecting incomplete recovery over a short period of refeeding.

There was no significant change in body weight in any group following rotavirus infection or immunization. Where animals were infected with rotavirus and immunized with picryl chloride a fall in body weight in all dietary groups was seen (chaper-6 and chapter-eight). It was not possible to establish if this was due to picryl chloride alone or to the mixed challenge. Since rotavirus challenge alone did not effect any weight loss, we assume the reduction of body weights were due to the use of Picryl chloride alone or a synergistic effect with rotavirus infection.

The effects of vitamin A deficiency and rotavirus infection were investigated in different organs. Liver was chosen since this is not a target tissue for the vitamin A action. Thymus and spleen were chosen because these are lymphoid organs and are a target tissue for the action of vitamin A.

As described the total liver weights of vitamin A deficient animals were significantly lower than those of control and pairfed animals. There was no significant difference in the relative liver weights of different dietary groups and rotaviris infection did not have any significant effect on liver weight. These findings were consistant throughout the study. Zile et al (1979) have shown similar results in vitamin A deficient rats.

With respect to spleen weight the results in different experiments were variable. In the preliminary study it was observed

that vitamin A deficiency was associated with a larger relative spleen weight (chapter-3). Following rotavirus infection, viamin A deficient animals had significantly higher spleen weights than control and pairfed animals (chapters-5 & 6). There was no significant change in the spleen weight of non-infected vitamin A deficient animals as compared to controls. Following intraperitoneal immunization there was no such increase in spleen weight. However, in this study irrespective of rotavirus immunization vitamin A deficient animals had significantly higher spleen weights as compared to control and pairfed animals (chapter-7). This variability reflects different treatments in the different studies performed. Smith et al (1987) have shown an increased spleen weight in vitamin A deficient mice and have suggested that the higher weight was due to the combined effect of vitamin A and inanition. These authors have also shown that raised spleen weight was due to the increase in B lymphocytes in the spleen. There was a significant reduction in the total but not the relative spleen weight in the diet restricted animals (chapter-nine). This result which occured irrespective of infection suggests that increased spleen weight is specifically associated with vitamin A deficiency alone.

There were no significant changes in the histology of the spleen between non-infected vitamin A deficient and control animals. An apparent increase in the number of splenic germinal centres in infected vitamin A deficient animals were observed as compared to control and pairfed animals (chapter-5). However, we could not demonstrate this increase by measuring the B and T cells areas following immunostaining (chapter-5). In practice it is very difficult to measure representative sections from all spleen samples. To compensate, the results were also expressed as a B/T cell ratio. Although there was a trend towards a higher B/T cell ratio in the vitamin A deficient mice the difference did not reach statistical significance.

There was a significant reduction in total and relative thymus weight in vitamin A deficient animals as compared to control and pairfed animals in this study. Rotavirus infection caused a

further reduction in the weight of the thymus in vitamin A deficient animals but not control and pairfed animals (chaper-5). When rotavirus and picryl chloride antigen were used simultaneously, the thymic weights of all groups were significantly lower than non-infected groups in the presence or absence of rotavirus infection (chapter-6). This result was also seen following mixed antigen challenge in the reduced food intake experiment (chapter-eight). It was not possible to establish if the reduction in thymus weight was due to picryl chloride alone or due to the mixed challenge. In this context we should note that Bang et al (1973) found that the thymus weight of vitamin A deprived chicks was normal until a stress such as infection was coupled with deficiency.

There were no significant changes in the histology of the thymus in either vitamin A deficient or control animals. However it was difficult to examine representative sections from the different animals and thus we could not reliablely compare the changes in the medulla and cortical area. Krishnan et al (1974) have reported atrophy of the cortical area in vitamin A deficient rats. Zile et al (1979) have reported that vitamin A deficient rats had significantly lower total and relative thymus weights compared with controls. These authors have also shown that vitamin A deprived rats had a deficit of one billion cells per gram of thymus compared with controls. Therefore, reduced thymus weight in our studies could reflect reduced cellularity.

In this project the EDIM strain of rotavirus was used throughout the study, was obtained from the Department of Microbiology, Birmingham University. Virus was obtained in three different batches. The activity of the virus suspension was determined by the donor and 30 µl viral suspension (contained $10^{4\cdot5}ID_{50}$) was capable of producing diarrhoea in infant mice (Starkey et al., 1986). After receiving the first and second batch of virus, the virulence was checked by examining the gut histology of infant mice 48 and 168 hours post-dosing (chapter-4). The histology results showed damage identical to that described by Starkey et al (1986) indicating that the

rotavirus we employed was virulent. Virus from the first batch was used in the first vitamin A deficiency experiment (oral challenge, chapter-5), intraperitoneal immunization (chapter-7) and in the moderate food restriction experiment (chapter-8). Virus from the second batch was used for oral challenge in the second vitamin A experiment (chapter-6). The third batch of rotavirus was used both for the oral and intramuscular immunization of animals in the severe food restriction experiment (chapter-nine).

In all these studies 30 µl/mouse of EDIM rotavirus was administered either orally, intraperitoneally or intramuscularly. We assume the amount of virus administered was more or less similar in each of the experiments. The variation in antibody production following rotavirus challenge in different experiments was not due to the use of virus from different batches since the different results obtained following virus administration by two different routes were achieved with the same batch of virus. This reflects the different routes of immunization employed (chapters-5 and 7) and the effects of different dietary regimens (chapters-5 and 8). Further oral challenge with rotavirus in two seperate vitamin A studies gave similar findings, even though two different batches of rotavirus were used (chapters-5 & 6). However, the levels of antibody in control animals in different experiments varied, presumably reflecting the different batches of animals used and also the variability of the antibody assay at different time points. When the data were compared within the same batch, however, the results were consistant.

In the present study following rotavirus infection only the middle portion of the small intestine was examined histologically. As Starkey et al (1986) have shown that the structural changes were confind to the small intestine, with the middle section showing the most pronounced changes. Further, they found no pathological changes in the colon of infant mice.

In vitamin A deficient animals without rotavirus challenge the villus tips were completely normal and similar to control. Vitamin A deficient animals showed a severe destruction of the villus tips in

the small intestine following rotavirus infection, whereas the villi of control and pairfed animals were normal. Thus in the vitamin A deficient animals the changes were specifically due to rotavirus. Animals fed a diet at 50 % less intake than the ad libitum control level also showed a significant change in villus histology. Following rotavirus infection of these animals it was not possible to demonstrate any further villus damage.

In infant mice there is clinical diarrhoea after 2-3 days of rotavirus infection (Starkey et al., 1986). We have also seen diarrhoea in infant mice. However, none of the vitamin A deficient, control or pairfed animals had diarrhoea. The mechanism of diarrhoea following rotavirus infection in infant is thought to be that the epithelium of the gut is destroyed including cells which synthesize disaccharidases. The lack of these enzymes affects the digestion of lactose or other disaccharides causing an osmotic drain, attracting body fluid into the bowel lumen (Flewett and Woode, 1978). The diet in the present study contained 20 % sucrose, a disaccharide, and therefore one might expect diarrhoea at least in the vitamin A deficient animals, since the extent of the villus damage in these animals was severe. Argenzio et al (1984) have investigated the consequences of a severe corona virus infection on small and large intestinal absorption in 3 day and 3 week old pigs using a non-absorbable marker, polyethylene glycol (PEG) incorporated in milk. Corona virus also causes damage to the small intestinal epithelium but not the colon. The results show that in 3 day old pigs, the PEG concentration was unchanged throughout the gastrointestinal tract indicating an absence of net absorption of fluid in both the small and large intestine. In contrast, 3 week old pigs with similar small bowel malabsorption showed that the marker PEG was concentrated 6 fold in the colon and this compensatory response prevented diarrhoea in the older animals. Graham et al (1984) have shown that following rotavirus challenge the colon of neonatal pigs did not show specific signs of infection. However, in the neonatal pigs the colon does not seem to be able to compensate with increased fluid absorption for the diarrhoea

produced by rotavirus. Thus the findings are consistant with those of Argenzio et al (1984), and with our findings in that following rotavirus challenge vitamin A deficient animals showed atrophy of the villus tips but had no diarrhoea. In fact the age dependent difference in the severity of diarrhoea in our study may be explained by the development of colonic function.

Control or pairfed animals escaped damage to the gut following rotavirus dosing, whereas a vitamin A deficient diet caused severe damage to the gut, demonstrating limited ability to withstand rotavirus. Gut immunity can be impaired non-specifically by lack of mucins and specifically by reduced secretory antibody production or both. Therefore, in this project investigations were carried out to evaluate the effect of vitamin A deficiency on both the non-specific and specific immnue system.

In order to investigate mucin production in the gut epithelium, the number of goblet cells in the villi of the duodenum were quantified. Goblet cells produce mucins and we assume the number of goblet cells in the villus would represent a reliable index of mucin production in the gut. Vitamin A deficient mice showed a significantly reduced number of goblet cells/villus as compared to control and pairfed animals (chapter-5), indicating defective non-specific immunity in the gut. This could be one of the reasons for the susceptibility to rotavirus infection of the vitamin A deficient mice.

In order to investigate specific immunity, serum antibody levels were measured following oral challenge with rotavirus. Vitamin A deficient mice showed significantly lower serum antibody levels as compared to control and pairfed animals (chapter-6). Lower serum antibody levels could parallel reduced local antibody secretion in the gut lumen and thus would imply impaired specific gut immunity. Sirisinha et al (1980) have shown that the local anti-DNP response was impaired in vitamin A deficient rats following oral immunization. But the systemic anti-DNP response was marginally affected in vitamin A deficient rats. This group have suggested that following oral

immunization, vitamin A deficient rats yield normal antibody levels but due to the reduction of secretory component the antibodies were not available in the gut lumen. In the present study, since the serum antibody levels following oral challenge were lower, we can expect lower antibody levels in the intestinal fluid. Again, if there is lack of secretory component in the gut epithelium, that would result in a further reduction of local antibody. In either case vitamin A deficient mice might be expected to have reduced specific gut immunity. Therefore, both non-specific and specific immunity is impaired in vitamin A deficient mice and as a result they become more susceptible to rotavirus infection.

When vitamin A deficient animals were fed a vitamin A supplement one week prior to infection, they showed more normal villus structure following the administration of virus which correlated well with a moderate but not statistically significant increase in mean serum antibody titre. Histologically it seems that vitamin A refeeding one week prior to infection is able to give partial protection against rotavirus infection suggesting that the immunological consequences of vitamin A deficiency can be reversed.

In the vitamin A deficient animals low serum antibody levels may reflect defective B , T or accessory cell function. In order to investigate T cell function the DTH response to picryl chloride was investigated. Vitamin A deficient animals showed a significantly impaired DTH response as compared to control and pairfed animals measured as a reduction in ear thickening in response to picryl chloride challenge. These results suggest defective T cell function. Smith et al (1987) have also shown an impaired DTH response even in the early stages of vitamin A deficiency. In the present study, refeeding of vitamin A to the deficient mice, one week prior to immunization, did not significantly alter the DTH response. This finding suggests that the refeeding time employed in this study may not be sufficient to return the DTH response to normal. The antibody levels following oral challenge were improved in the refed animals, suggesting that the humoral and cellular immune system have different

vitamin A requirements for normal function.

The results reported in this thesis lead to the conclusion that vitamin A deficiency increases susceptibility to rotavirus infection in mice. Increased susceptibility is due to impaired gut immunity. Further, the impaired gut immunity probably results from defects in both non-specific and specific host defense mechanisms which combine to increase the severity of rotavirus infection. The impairment of non-specific immunity resulting from an alteration in the epithelial barrier and lack of secretory antibody in the gut would allow more virus to attach to the epithelium and as a result increase the severity of rotavirus infection.

The present study opens several areas for further investigation:

a) Is the time course for the development of vitamin A deficiency shorter in the presence of other specific deficiencies, e.g. protein or zinc or in the presence of increased gastrointestinal losses such as parasitic infections ?

b) Is the lymphocyte traffic in the vitamin A deficient mice normal ?

c) Is there any defect of antigen presentation in the gut of vitamin A deficient mice ?

d) Is the proliferation rate of B and T cells of the Peyer's patch and mesenteric lymph node normal in response to antigen or mitogen ?

e) Are there changes in the levels of manufacture and secretion of secretory component in these animals ?

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APPENDIX-I

Calculations for the vitamin A estimation

The equation used for the calculation of vitamin A concentration in serum or plasma is based upon measurements of O.D. at 620 nm and the factors relating to recovery, carotene concentration, and the necessary conversion of the vitamin A acetate standard curves to their alcohol equivalents.

Let K_1= recovery factor,

K_2= conversion factor for relating acetate standard curves to alcohol equivalent,

K_2= MW alcohol/MW acetate =286/328 =0.872,

C = O.D. 620 nm contribution of carotenes

C = is equivalent to (Z/X)Y,

where Z is a variable (µg carotene/100 ml serum) and X and Y are constants, where X= 100 µg/100 ml with an O.D. 620 nm of Y=0.016.
but since X and Y are constants, we can let $Y/X = K_5$,
therefore, $C= K_5 \cdot Z$,
$Y = Mx + b$, the linear equation expressing the Beer's law relationship (µg/100 ml vitamin A to O.D. 620 nm) can be reduced to $Y= Mx$.
where, Y= O.D. at 620

x= µg/100 ml vitamin A

M= slope,

thus, $x= Y/M$ and allowing for the corrections above,

$x= K_4(Y-C)/M$

where, $K_4=K_1 \cdot K_2$

x= actual vitamin A (µg/100ml)

APPENDIX-II

Calculations for antibody assay

The units for anti-rotavirus activity are defined for the standard serum as dilution of serum X ($1x10^6$). The antibody units were converted into Log_{10} and the standard curve (Fig-2.7) was plotted = log_{10} versus Absorbance units at 490 nm.

Therefore, if a test serum showed an O.D.=0.67 at 1/16 dilution then the corresponding log_{10}= 1.8
Units of rotavirus antibody = anti Log of 1.8 x 16
Therefore, actual antibody levels= 1010 units.