Vitamin A Status and Susceptibility to Respiratory Illness

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Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma at any University or, to the best of my knowledge and belief, the thesis contains no material previously published or written by another person, except where due reference has been made in the text of the thesis.

I consent to this thesis being available for photocopying or loan.

Carole B.Pinnock

For

Geoffrey and Stephanie Pinnock

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Abstract

1. Vitamin A is known to have a number of functions and effects which are related to susceptibility to infection, particularly of the respiratory tract.

2. No previous systematic attempt appears to have been made to investigate whether vitamin A status is a determinant of respiratory morbidity in Western populations or, in particular, is causally significant in individuals prone to respiratory infection.

3. The 1-5 year old age group experiences peak respiratory morbidity, and this is also a time when vitamin A reserves can be expected to be low. A randomized controlled trial of vitamin A supplementation was carried out in a group of 147 preschool children who had experienced frequent respiratory symptoms in the preceding 3 months. A 19% drop in number of episodes, but not days of symptoms, was associated with supplementation. The difference was observed particularly amongst children with a history of lower respiratory infection. Children who experience lower respiratory infection in infancy have been shown in many studies to experience higher than normal levels of respiratory morbidity later in life and this group was selected for further study.

4. A group of 206 2-6 year old children who had been hospitalized in infancy with respiratory syncytial virus positive bronchiolitis were identified. A randomized, controlled trial of vitamin A supplementation in

this group showed no effect on respiratory symptoms. However plasma vitamin A values were unusually low and did not change with supplementation. The relationship between vitamin A status and respiratory morbidity in this group, particularly in the first year of life, and the reason for the apparently low plasma values which did not respond to supplementation, remain to be investigated.

5. Plasma retinol levels are independent of dietary intake and liver stores in well-nourished persons. A tissue based assessment of vitamin A status based on the vitamin A content of buccal mucosal cells was developed in conjunction with the second study.

Chapter 1

Introduction

Respiratory illness is a major source of morbidity and mortality in both the developed and developing world . Acute respiratory illness is thought to be the cause of a quarter to a third of all deaths in children under 5 years in many countries of Africa, Asia and Latin America (Pio et al, 1985), while in Australia, 4 times as many people of all ages die from respiratory infections than from all other communicable diseases combined (Douglas, 1985). The burden of morbidity is correspondingly high. A longitudinal study of 1707 residents of Manhattan found that 60% of illness and 81% of person-days of illness were due to respiratory illness and, depending on age, persons were ill 25% to 60% of the time (Lebowitz et al, 1972 a,b). Much of this illness is untreated or untreatable. Viruses, for which there are few effective treatments, are the major cause of respiratory infections in developed countries (Mufson, 1985). A recent conference on acute respiratory infections in childhood reported that even known treatment measures were not being applied in some cases and pinpointed effective case management as a major priority for the developing world (Pio et al, 1985; Final Conference communique, 1985).

An absence of effective treatment for upper respiratory illness has focused attention on potential preventive measures as a means of control. The interaction between nutritional status and infection has been demonstrated in many studies (Scrimshaw, 1968; Suskind, 1977; Chandra, 1977; Chandra, 1983, Stinnett, 1983, Tupasi, 1985). That such an interaction is complex was demonstrated by Scrimshaw (1968), who reviewed studies showing both synergistic and antagonistic interactions

between malnutrition and infection, depending on the nature of the invading organism and the host species. In virtually all human studies, however, malnutrition increased severity or had no effect. Thus, Tupasi (1985) found an increased case-fatality rate from acute respiratory illness in children with severe protein-calorie malnutrition, but not an increased incidence of the disease. Barclay et al (1987) reported that mortality from measles was several times higher amongst marasmic than better nourished, low weight for age children.

An interaction between nutrition and infection is of particular public health importance in developing countries. However it may also play an important role as a contributor to morbidity in affluent countries. Overnutrition as well as undernutrition can result in deficits in the immune response (Chandra, 1981, 1977). Nutrition may play a role in the immuno-competence of nutritionally at risk groups, such as the newborn and the elderly (Watson, 1984). Low birthweight infants are at nutritional risk at a critical time in the development of their immunocompetence (Chandra, 1983). Chandra and Puri (1985) found an improved response to influenza vaccination amongst elderly with nutritional abnormalities, when those abnormalities were corrected with nutritional supplementation. The effects of infection itself on the metabolic state of the host (Chandra, 1977; Beisel, 1979, 1984) and the differences in metabolic consequences of disease-induced malnutrition as compared with starvation-induced malnutrition (Beisel,1984), suggest that frequently ill persons in a community may be at particular risk of a nutrition-infection interaction.

Few studies have investigated the influence of diet on morbidity and

mortality due to acute respiratory illness in Western communities, however. A study on this subject, with particular importance in initiating the present research was conducted by Douglas and Muirhead (1983) .The community investigated was the readership of the magazine Australian Women's Weekly, a popular magazine with a large, nation-wide circulation. A questionnaire which sought information on social, psychological and environmental factors associated with acute respiratory infections was published in the magazine. The questionnaire collected information on members of the family who were most and least prone to respiratory infections, as well as the respondent. A total of 3495 questionnaires concerning 8470 individuals were returned. A question on the type of diet showed 7213 persons to have a normal diet, 322 individuals to have a vegetarian or high fruit and vegetable diet and 642 persons who had other specified "special diets", and this was analysed in relation to number of respiratory episodes during the preceding 12 months. The vegetarian and high fruit and vegetable group had consistently fewer episodes of respiratory illness over all age groups (age adjusted mean 1.95 episodes during the preceding 12 months, sem 0.16 compared with 2.53 episodes, sem 0.03 respectively). Fewer of the high fruit and vegetable eaters (25.7%) reported a tendency for colds to "go to the chest" compared with the normal diet group (29.5%). Individuals who supplemented their diet with daily orange or lemon juice did not differ from those on normal diets with respect to respiratory infections, while those who supplemented with vitamin C reported higher mean episodes of infections.The families who returned questionnaires are not necessarily representative of all Australian families. However the consistency of these observations within the study population. and particularly their consistency over all age groups,

suggested the possibility that vegetable eating was protective for acute respiratory infections. If so, the question was raised with respect to the current research, whether there were any constituents of vegetables, which are not also major constituents of fruit, which have known functions that could provide a theoretical basis for expecting a protective effect for acute respiratory infections.

A subsequent study of the literature identified vitamin A as a nutrient with known functions which could be directly related to resistance mechanisms for respiratory infection. The role of this vitamin in the maintenance of secretory epithelia of the respiratory tract and its effects on cell-mediated immune function are well documented and described in chapter 2 of this thesis. They provide a basis for expecting an interaction between vitamin A status and susceptibility to infection to occur, at some point in the continuum between optimal availability and clinical deficiency.

Vitamin A deficiency is widespread throughout the developing world. A recent meeting of the World Health Organization estimated a prevalence of 250,000 cases of severe deficiency with blindness in 4 major Asian countries, and as many as 8-9 million cases of mild deficiency (WHO, 1982,1985). In Western countries, vitamin A deficiency is not a significant health problem, although surveys in the U.S.A. and Canada showed some groups to be at moderate risk (Underwood, 1984). Low income groups such as Mexican Americans, black Americans in low income states and Indians on reservations had low serum vitamin A levels in the Ten-State Nutrition Survey in the U.S.A., 1972. Age as well as socio-economic status

is a determinant of vitamin A status. Vitamin A reserves are low in neonates and subsequently increase, reaching adult levels by the age of 4 years (Olson et al, 1984). Vitamin A levels are lower in premature than full term infants (Shah and Rajalakshmi, 1984; Howells et al, 1984). Amongst premature infants, Shenai et al (1985) reported plasma vitamin A levels were lower in those who subsequently developed bronchopulmonary displasia than in healthy controls of the same gestational age, and remained low for 28 days postnatally. Dietary intake of Australian children generally has been reported as adequate (Commission of Inquiry into Poverty, 1975; Stuckey and Darnton-Hill, 1980), although studies of sub-groups who may be at risk, such as aboriginals, the chronically ill and the elderly are lacking.

The age at which clinical vitamin A deficiency is most prevalent, 1-4 years (Sommer, 1983; Tielsch and Sommer, 1984), is also the age at which respiratory infections reach their peak (Buck, 1956; Dingle et al, 1964), and thus provides a basis for expecting an interaction between the two to be in this age group.

Despite the general interest in nutrition and susceptibility to infection, few studies have examined the role of vitamin A deficiency as a contributor to infectious morbidity, probably because of the difficulty of separating its effects from those of protein-energy malnutrition. Sommer, in three recent studies in Indonesia (1983, 1986a, 1986b), reported the association of vitamin A deficiency with increased mortality from all causes in the preschool age group and with increased morbidity due to diarrhoea and respiratory infections (section 2.4.2). The difficulties encountered by

these workers illustrate why very little information is available oN the impact of marginal vitamin A status on respiratory morbidity, even in countries where vitamin A deficiency is endemic and morbidity and mortality due to respiratory infections is high. No information is available about such an interaction in Western communities. The following chapters describe an attempt to obtain preliminary information on this relationship in a population of pre-school age children, resident in the suburbs of Adelaide.

Chapter 2

The Biology of Vitamin A: Definition and Sources of Variability in Vitamin A Status

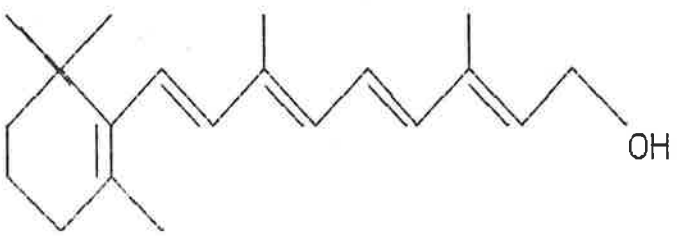
2.0 Introduction

The following chapter describes uptake, transportation, storage and mobilization of vitamin A and the way in which variability is introduced into these processes. The concept of vitamin A status, its relationship to these factors and the use of the term in this thesis is then discussed. Subsequently the way in which vitamin A status may interact with infection in determining susceptibility to further infection is described.

2.1 Metabolism of Vitamin A

2.1.1 Structure and Natural Sources of vitamin A

Vitamin A has been defined by theIUPAC-IUB as a generic descriptor for all compounds comprising 4 isoprenoid units joined in head to tail manner (termed retinoids) which have the qualitative biological activity of retinol (Nomenclature Policy,1984). These molecules have in common a conjugated double bond system, which confers 2 important properties: a high extinction coefficient necessary for its role in vision, and extreme hydrophobicity- a property which plays a major role in determining how retinol is handled by the body (Ong, 1985). Vitamin A is essential for normal growth, reproduction, vision and maintenance of epithelial surfaces. The most physiologically active, naturally occurring form of vitamin A is retinol:



Recent advances in organic chemistry have enabled over 1000 retinoids of varying biological activity to be synthesized. These have been used extensively in therapeutic dermatology, oncology and in experimental studies of cellular differentiation and proliferation. The development of forms of vitamin A, the biological activity of which exceeds that of retinol, has prompted some workers to suggest an alternative definition of vitamin A based on binding specificity rather than chemical structure (Sporn and Roberts, 1985).

Mammals are not capable of synthesizing vitamin A de novo, but must rely on dietary sources. Dietary vitamin A is derived indirectly from intestinal conversion of dietary provitamin A compounds. The latter compounds are red and yellow, fat soluble pigments, called carotenoids. Carotenoids are structurally related to vitamin A and are present in all photosynthetic tissues. Of the carotenoids, ß-carotene is the form most efficiently converted to vitamin A and the one found in greatest quantities in green and yellow vegetables and yellow fruits. Other forms, α and γ carotene and their derivatives, the xanthophylls are less significant dietary sources of vitamin A (Underwood, 1984). Preformed vitamin A (mainly in the form of long chain fatty acid esters of retinol) is converted from provitamin A compounds by herbivores and secreted in milk. The major dietary sources of retinol are therefore milk, cheese and high-fat dairy products. Western diets provide approximately 50% of vitamin A as preformed and 50% from provitamin A sources.

NOT SOURCES OF VITAMIN A) ONGENATED

Retinol, the most active form of vitamin A is a highly reactive, polar molecule, toxic in its unbound form, which is transported in the body bound to protein (retinol transport proteins serve both a

protective and regulatory function). Vitamin A is stored mainly in its esterified form (retinyl palmitate). which is less polar and transported in combination with lipids in chylomicrons. Exchange between these two forms occurs frequently as vitamin A is transferred between water and lipid-soluble fractions of the cell and plasma during the process of uptake, storage and mobilization.

2.1.2 Absorption

Events taking place during the absorption of vitamin A have been most recently reviewed by Goodman et al (1984). Intestinal absorption of vitamin A is closely associated with that of dietary lipids. Hydrolysis of intestinal lipids (triglycerides, cholesterol and retinyl esters) by pancreatic enzymes takes place in the lumen of the gut and the products of lipolysis are emulsified with the aid of bile salts to form mixed micelles. These particles (containing a lipophyllic core of fatty acids, monoglycerides, bile salts, cholesterol and fat-soluble vitamins, and a hydrated outer surface) diffuse across the stationary aqueous layer to the mucosal surface, where they are taken up by a process of passive diffusion. This is a saturatable process (Hollander, 1981) and there is evidence of competitive antagonism between fat-soluble vitamins at high concentrations (Weber,1981).

Factors which affect the partitioning between the oily, micellar and luminal cell membrane phases affect the uptake of fat-soluble vitamins. Production of bile salts enhances this process by activating hydrolytic enzymes and promoting micellar formation (Weber 1981, Kahan 1970). Solublizing factors in the diet, such as lecithin and fatty acids

may also assist (Moore 1957, Hollander 1981). Dietary polyunsaturated fatty acids, however, tend to decrease uptake of fat-soluble vitamins (Ames 1969, Weber 1981), possibly by stablizing micelles and reducing uptake by intestinal cells. Although a high dietary fat content promotes vitamin A uptake efficiency by increasing the size and number of mixed micelles (Weber 1981), any factors (such as reduced stomach acidity) which are likely to increase the concentration of unhydrolysed triglyceride in the gut will reduce efficiency of absorption.

Antioxidant activity in the gut may also increase uptake of retinol. Simultaneous administration of vitamin E with oral supplements of vitamin A have been shown to improve utilization, although it is not clear whether this is due to enhanced absorption or post-absorptive effects (Jagadeesan and Reddy, 1978).

Efficiency of absorption of retinol has been reported as 30-80% (Weber 1981), and that of ß-carotene as approximately 33% (Underwood, 1984), depending on the factors described above. Although it is unlikely that changes in absorption efficiency will affect vitamin A status in children with adequate liver reserves, variability in efficiency of uptake of vitamin A may be a significant factor in determining vitamin A status in those with marginal stores (Roels et al, 1963).

2.1.3 Transportation to and Uptake by Liver

Within the intestinal mucosa, ß-carotene is cleaved to retinaldehyde and further reduced to form retinal. Retinol from this source and from dietary preformed vitamin A is then re-esterified and incorporated into

chylomicrons which are secreted into lymph and join the general circulation via the thoracic duct (Goodman and Blaner, 1984). Small amounts of retinol and retinoic acid are transported to the liver via the portal vein (Ganguly, 1969), however the major portion of dietary retinol is carried in chylomicrons. Retinyl esters are transported mainly bound to lipoprotein in the VLDL fraction, however substantial amounts of retinyl palmitate may be associated with LDL (Schindler and Klopp, 1985) and may possibly play a role in transporting vitamin A to peripheral tissues. Transport of retinyl esters is reduced in patients with abetalipoproteinemia (Goodman and Blaner, 1984).

Chylomicron remnants are taken up by the parenchymal cells of the liver and retinyl esters are hydrolysed. Retinol may then be reesterified and stored in fat droplets within the parenchymal cell or transferred to fat-storing non-parenchymal cells elsewhere in the liver (these are thought to act as a storage sump). Alternatively, retinol may combine with retinol binding protein and be released into the general circulation. Which of these pathways is taken, depends on the vitamin A status of the individual. If circulating levels are low, most newly absorbed vitamin A is mobilized (even if liver stores are low). If circulating levels are adequate, most retinol is reesterified and stored, although a portion of newly absorbed retinol is still thought to be mobilized (Underwood, 1984)

2.1.4 Plasma Transport of Vitamin A

Vitamin A is transported in plasma bound to retinol binding protein, a low molecular weight protein, synthesized by liver parenchymal cells and combined with retinol before secretion into the blood stream. Plasma

retinol levels are determined by the rate of synthesis of retinol binding protein under conditions of dietary sufficiency, and are regulated at a homeostatic set point (Loerch et al 1979), although dietary intake may fluctuate widely. The retinol-RBP complex associates in plasma with a high molecular weight protein called transthyretin, which also transports thyroid hormones. A minor proportion of retinol is transported associated with various serum lipoproteins (Schindler and Klopp 1985, Ellis et al 1986, Schindler et al 1985).

Retinol is delivered to tissues via specific cell surface receptors for RBP (Ong, 1985) and, after loss of its ligand, circulating apo-RBP dissociates from transthyretin. The smaller molecular weight protein is filtered and excreted by the kidney. Apo-RBP is not recycled through the liver (Goodman et al 1984), however apo-RBP may play a role in stimulating de novo synthesis of RBP in response to increased tissue utilization (Loerch et al 1979).

Plasma retinol levels drop when tissue utilization rates are reduced, when liver reserves are exhausted because of inadequate dietary intake, and in response to a number of physiological influences. Changes in steroid hormone secretion which occur during the menstrual cycle, or as a result of oral contraceptive use, change the homeostatic set point (Underwood, 1984). Plasma levels increase in response to estrogen secretion, probably due to stimulation of hepatic RBP synthesis. Increased secretion of adrenocortical hormones due to stress (physiological, emotional or dietary) leads to a net lowering of plasma retinol levels in vivo (Underwood,1984). Conditions which affect protein synthesis (such as protein-energy malnutrition) or any of the enzymatic processes involved in

the hydrolysis and reesterification of retinol (such as zinc deficiency), will affect plasma retinol levels.

2.1.5 Intracellular Distribution of Vitamin A

Intracellular retinol is found in the cytosol bound to a small molecular weight polypeptide, cellular retinol binding protein (CRBP). CRBP and closely related cellular retinoic acid binding protein (CRABP) have been found in almost every organ of the body (Ong, 1985). Several other transport proteins which are tissue specific have been found in the eye, intestine and testes. The intracellular metabolic function of retinol is unresolved, however CRBP has been shown to transfer retinol to specific binding sites on the nucleus. The number of such sites has been shown to be dependent on vitamin A status (Ong,1985).

2.1.6 Excretion

Retinol is excreted in bile as polar conjugates, some of which are reabsorbed in an enterohepatic circulation (Anonymous, 1985a). Retinol is catabolized irreversibly by oxidation to less active forms. Retinaldehyde retains most of the biological activity of retinol, but its oxidation product, retinoic acid, while supporting growth and differentiation of epithelial tissue, cannot function in vision or reproduction (Goodman, 1980). Oxidation products of retinol are excreted by the kidney as well as by the liver (Underwood, 1984).

2.1.7 Definition of Vitamin A 'status'

Underwood (1984) refers to nutritional status of a nutrient as the difference between uptake, utilization and excretion of that nutrient over a period of time. Vitamin A status is the 'sum product over time of the dynamic processes involving uptake, storage, mobilization, utilization and excretion' (Underwood, 1984). This would imply that at any one point, vitamin A status is most closely reflected in liver reserves. However liver reserves do not necessarily reflect availability of vitamin A at the cellular level. For many nutrients, the latter is closely related to dietary intake and total body stores. For vitamin A the distribution of retinol to tissues is highly regulated. Many factors determine the rate of release from liver (section 2.1.3, 2.1.4), however total body stores does not appear to be among them. Uptake by tissues is also a controlled process influenced by factors which are at present, largely unknown. The factors which influence the level of vitamin A at the point of utilization are therefore many, and in addition to those affecting intake and total body stores.

Vitamin A status is thus defined, and used subsequently in this thesis, as a state or condition which results from the sum of the processes of intestinal uptake, storage, mobilization, transportation and cellular uptake of vitamin A and which determines the presentation of vitamin A in optimal levels to meet requirements at the cellular level.

Conventional wisdom states that plasma retinol is not a sensitive index of vitamin A status in marginally or well-nourished persons, because it is independent of liver stores. Under the broader definition, we note that it is not a sensitive index of vitamin A status

because it does not vary closely with functional indices such as cellular retinol binding protein (Anonymous 1985b), dark adaptation (Solomons et al, 1982) and risk of epithelial cancer (Friedman et al, 1986).

2.2 Effect of Disease States on Vitamin A Status

In addition to physiological variables, a number of disease states affect the processes described above. Those which affect pancreatic function, (such as cystic fibrosis) or biliary secretion,(such as cholestasis) reduce absorption, as do conditions which directly affect the lining of the intestine(such as celiac disease, diarrhoea, parasitic infestation, tropical or non-tropical sprue or short bowel syndrome)(Amadee-Manesme et al, 1985, Mahalanabis et al, 1979, Krishnan and Krishnan 1976 and Underwood, 1984).

Liver disease of various kinds (eg cancer, infectious hepatitis, alcoholic cirrhosis) is frequently associated with low plasma retinol levels, probably due to reduced mobilization of retinol from stores (Underwood, 1984). Drug administration has been associated with low plasma retinol levels and low liver stores of vitamin A, possibly due to enhanced catabolism of retinol (Leo et al, 1984). Segawa et al (1986) reported lower plasma retinol levels for patients with pancreatic cancer, calcified pancreatitis, inflammatory bowel disease and liver cancer.

Conditions which increase metabolic rate, such as hyperthyroidism, febrile and afebrile acute infections and stress, are also associated with low plasma retinol levels (Underwood, 1984). The drop in plasma retinol with

infection is quite marked and persists for some time (Arroyave and Calcano, 1979; Bhaskaram et al, 1986). It is associated with a drop in circulating retinol binding protein, but the mechanism for the rapid change is not known (Anonymous, 1981). Sequestration of iron, another acute phase response during infection, is thought to have benefits in inhibiting bacterial growth (Beisel, 1979). The potentially adaptive role of changes in plasma vitamin A during infection has not been considered in the literature.

Infection may also affect vitamin A status generally through decreased absorption (Sivakumar and Reddy,1972; Mansour et al,1979 and Mahalanabis et al, 1979) and increased utilization secondary to the anabolic response and tissue repair (section 2.4.1)

2.3 Physiological Functions of Vitamin A

Vitamin A is involved in a wide range of physiological processes: growth, reproduction, hematopoiesis, bone deposition, fetal growth, vision and maintenance of epithelial and mesenchymal tissue. Deficiency in experimental animals causes elevation of cerebro-spinal fluid pressure, slowing of growth, histological changes in mucosal structures, defects in cell mediated immunity,dark adaptation, xerosis and xeropthalmia. The presence of a cellular retinol binding protein in almost every organ system attests to the universality of vitamin A function. Whether this is achieved through regulation of cellular differentiation and proliferation or other mechanisms is unknown (Sporn and Roberts 1985, Anonymous, 1985c). The exact biochemical pathways involved, and the mode of action in controlling gene expression, remain to be determined.

The functions most closely related to susceptibility to respiratory illness relate to immunity and maintenance of mucosal surfaces. The function of vitamin A in vision illustrates how closely the structural and functional aspects of vitamin A activity are intertwined. and how they both interact in determining susceptibility to infection.

2.3.1. Vision

The role of vitamin A in vision is one of its earliest known functions. It has a role in maintaining both the structural integrity and normal function of the eye. Vitamin A is involved in the synthesis of complex polysaccharides. important in maintaining the integrity of the cornea. the wettability of the corneal conjunctival epithelium. tear duct function and lysozyme production (reviewed by Inua.1983).

Vitamin A deficiency results in conjunctival xerosis (stage X1A. WHO.1976) . Bitot's spot formation. and subsequently corneal xerosis (stage X2). Drying of the cornea is followed by loss of substance of part or whole of the corneal thickness (WHO.1976). Keratolmalacia - total loss of structure of the cornea and extrusion of the lens - is the end stage of deficiency. This process is often accompanied by local infection and the extent to which changes are due to vitamin A deficiency or infection. or a synergistic interaction between the two. is not clearly determined (section 2.4.1). An interaction between vitamin A deficiency and the severity of histopathology of the eye in experimental Herpes simplex infection has been shown by Nauss et al (1985a.b). Simlarly, vitamin A deficiency and local infection may interact to enhance corneal lesions resulting fom

measles virus infection (Bhaskaram et al.1986).

The role of vitamin A in night blindness was one of the first described functions of the vitamin (Wald. 1935).The photosensitive retinoid 11-cis-retinaldehyde is the prosthetic group of the light sensitive pigment, rhodopsin. Excitation with light converts the cis form to all-trans retinaldehyde. subsequently reduced to free all-trans retinol. The process of recovery of cis-retinaldehyde is not completely understood (Bridges, 1984). The absorption maximum of rhodopsin gives it maximum sensitivity in dim light. Recovery of vision in dim light. after bleaching (dark adaptation). forms the basis of a sensitive test of vitamin A availability.

Vitamin A is stored as retinyl esters (both 11-cis and all trans forms) in retinal pigment epithelial cells. Considerable quantities of vitamin A are stored in this way, however this does not appear to be a potential source for the rest of the body and is retained during vitamin A deficiency (Bridges, 1984). It is interesting that all special sensory cells (eg olfactory, gustatory, hair cells of the inner ear) are dependent on vitamin A for normal function and store relatively large amounts (Chole 1978. Biesalski et al 1986). All such cells contain cilia or ciliary remnants and demonstrate a common effect of vitamin A deficiency on their structure and function.

2.3.2 Immunity

Evidence for involvement of vitamin A in major aspects of the immune response is considerable. Beisel. in a comprehensive review (1979). cited

both enhancement and reduction of humoral and cell mediated immune function in response to vitamin A administration and deficiency respectively. Antibody responses were diminished in vitamin A deficient rats and chicks. and numbers of splenic plaque forming cells reduced. T-cell transformation in rats was also reduced and restored with vitamin A supplementation. Reduction in delayed dermal hypersensitivity response and enhanced skin-graft rejection were other consequences of vitamin A deficiency reported in this review.

Later reports have been consistent with this work. Enhanced induction of antibody forming cells and antibody production occurred in human tonsillar lymphocyte cell lines (Sidell et al. 1984) and in mice and rabbits (Pletsityi and Askerov, 1982) in response to vitamin A administration. High doses of vitamin A (50,000 iu), given to patients with chronic pneumonia resulted in increased numbers of T and B lymphocytes as well as increased serum IgG and IgM. Mitogenic response to PHA was enhanced (Pletsityi et al. 1982). Kitano observed similar effects in rats (1985). Pletsityi (1985) concluded that vitamin A may block the generation of antigen specific suppressors. Anti-immunosuppressive activity was also reported by Nuwayri-Salti and Murad (1985).

Soppi and Lehtonen (1984) reported that different retinoids selectively activate different components of the immune response Thus retinoic acid stimulated mainly Igm antibodies, while its derivative TMMP-retinoic acid increased IgG antibodies. Both affected response to T-dependent, but not T-independent antigen.

Mucosal immunity is particularly important in respiratory infection

Bang (1972) found that vitamin A deficiency in chicks reduced lymphocyte populations in submucosal tissue. Infection with Newcastle disease virus resulted in 100 times more virus being collected from throat swabs of deficient chicks compared with normal controls. Sirisinha et al (1980) reported lower secretory IgA levels in the intestinal fliud of vitamin A deficient rats, while serum levels were unaffected, and attributed the effect to reduced synthesis of secretory component. Mildly vitamin A deficient rats, infected ocularly with Herpes simplex virus, showed reduced cervical lymph node (and splenic) lymphocyte transformation and natural killer cell cytotoxicity (Nauss and Newberne, 1985; Nauss et al, 1985a,b). Disease onset was more rapid and severe than in pair-fed controls resulting in epithelial ulceration and necrosis. Vitamin A deficiency was not severe enough to affect growth in these animals, nor were systemic antibody titres affected.

Non-specific immune responses are also modulated by vitamin A. Goldman (1984) reported enhanced phagocytic activity in macrophage-like cell lines in response to retinoids. Retinoic acid was more potent in this regard than retinol or retinyl acetate. Rhodes and Oliver (1980) found suppression of phagocytic activity with vitamin A deprivation in human macrophage cell lines. Vitamin A supplementation increased the bacteriacidal activity of neutrophils in humans, particularly lung cancer patients, although it did not affect their ability to capture microbes (Davydova et al, 1985). Smith et al (1986) found that topical vitamin A increased wound-healing in cortisone treated rats. The mechanism proposed for this action was an enhancement of the inflammatory response, possibly by lysosomal labilization.

Enhanced resistance to infection by Listeria monocytogenes was reported by Hof (1977,1979), after intraperitoneal or oral administration of high (toxic) levels of vitamin A. Cohen and Elin (1974) also reported enhanced host resistance with administration of lower amounts of vitamin A in mice infected with Pseudomonas aeruginosa, Listeria monocytogenes and Candida albicans. Vitamin A deficiency has been reported to affect host resistance to protozoa and other parasites: vitamin A deficient rats innoculated with Plasmodium berghei developed more severe parasitemia than pair fed normal controls (Krishnan and Krishnan 1976).Darip et al (1979) attributed more severe infection with Angiostrongylus cantonensis in vitamin A deficient rats. to altered penetrability of the intestinal mucosa.

This summary does not attempt to be a comprehensive review of recent work on the immunoregulatory effects of retinoids. This subject and the effects of vitamin A on tumorigenesis have been reviewed comprehensively by Vyas and Chandra (1984). Nevertheless, the diversity of effects, encompassing antibody production, T-cell function, phagocytic activity and other non-specific aspects of immune function is remarkable. Nauss (1985a) reported that effects of vitamin A deficiency on cell-mediated immunity occurred early during deprivation, before effects on growth could be detected. It seems reasonable to expect an interaction between vitamin A status and resistance to naturally acquired infection and for this effect to occur at levels of vitamin A status which precede signs of clinical deficiency in marginally nourished subjects.

2.3.3 Cellular Differentiation and Proliferation

Many of the physiological effects of vitamin A may relate to its role in controlling cell differentiation and proliferation. These effects were first reported by Wohlbach and Howe (1925) who recorded replacement of columnar epithelium with squamous, keratinizing cells in vitamin A deficient rats. Subsequently, the effects of retinoids on many different tissues - of ectodermal, endodermal and mesodermal origon - have been demonstrated. Loss of goblet cells and squamous metaplasia of epithelium with vitamin A deficiency, has been reported for cornea, conjunctiva, gastrointestinal, urinary and genital tracts, salivary gland and pancreatic ducts (Olson,1972; Underwood,1984) and in the respiratory tract, middle ear and eustachian tube epithelium (Chole,1979) and trachea (Wong and Buck,1971). Because of the striking changes in differentiation, such cell systems have become the basis for a number of assays of biological activity of retinoids (Sporn and Roberts,1984). Such systems are sensitive to small changes in vitamin A availability. Clark and Marchok (1979) found that the quantity and characteristics of mucin produced in rat tracheal organ culture was changed in deficient animals, well before histological changes were apparent. Zile et al (1981) found a reduction in DNA labelling index in trachea of rats while growth was still normal, although liver reserves were depleted. Biesalski et al (1986) found a reduction in numbers of ciliated cells with depletion in guinea pig epithelium, before squamous metaplasia was evident.

Retinoids are thought to exert their effects by controlling gene expression, through direct action on the cell nucleus (Roberts and Sporn, 1984), however the mechanism by which this happens is poorly

understood. Roberts describes the effects of retinoids as a 'switching action' - the turning on of a succession of events. the nature of which depends on the cell system in which they are operating. Thus in some systems, retinoids stimulate growth, while in others they suppress it. This tissue specificity of effect has led some workers to propose that retinoids be defined on the basis of their ability to combine with a particular receptor, rather than according to their chemical structure (Sporn and Roberts, 1985).

The action of retinoids in inhibiting neoplastic transformation and in promoting differentiation of fully transformed cells has stimulated intense interest in the potential therapeutic role of retinoids.
The role of vitamin A in carcinogenesis has been reviewed recently by Olson (1986).

2.4 Interactions Between Vitamin A and Infection

Although experimental evidence of modulation of the immune response gives us a basis to expect an interaction between vitamin A status and infection, it cannot tell us what impact such an interaction would have on the probability of aquiring infection or how it would determine the course of naturally acquired infection. This section looks at evidence of a modulating effect of vitamin A status on the course of naturally or experimentally acquired infection. Such an interaction could take two forms:

1. modification of the effects of infection by poor vitamin A status.
2. modification of the effects of poor vitamin A status by infection.

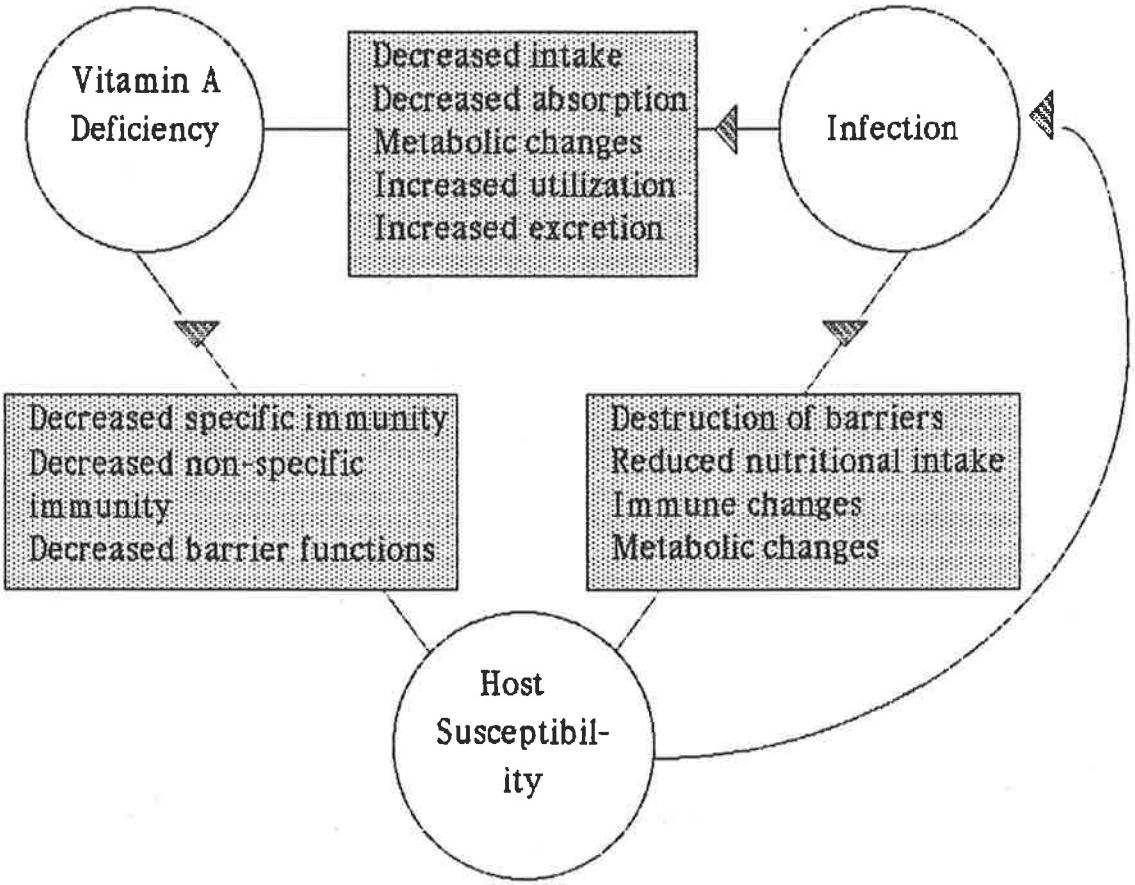
There is evidence that both types of interaction occur. When present simultaneously, both types of interaction are likely to be cyclical, leading to a rapid deterioration in health. Both interactions are probably of public health significance in developing countries where both low vitamin A status and frequent infection are prevalent, although coexisting malnutrition makes this a difficult problem to assess. In developed countries, where vitamin A deficiency is virtually unknown, the pattern of interaction is likely to be different. Infection is unlikely to precipitate deficiency when vitamin A status is good. However the level of vitamin A availability at which interaction occurs and other mediating factors, notably the presence of other conditions which affect vitamin A status (see preceding sections), may determine whether vitamin A status in turn affects incidence of infection in these populations. The ways in which vitamin A status and infection may affect host susceptibility to further infection are summarized in figure 2.1.

2.4.1 Effect of Infection on Vitamin A status

Infection has long been known to influence nutritional status (Brown and Black.1981: Scrimshaw 1968) and to contribute to malnutrition in disadvantaged populations (Mata et al.1972,1977; Martorell.1980). Anorexia, decreased absorption of nutrients, increased catabolic losses and alterations in metabolic pathways are postulated mechanisms (Brown and Black. 1981). Vitamin A status in particular is affected in this way (section 2.1.2.2). It would thus be expected that infection would precipitate overt symptoms of deficiency, such as epithelial keratinization, xerophthalmia and death in hosts whose vitamin A status is marginal. Vitamin A deficient rats in a germ free environment survive

Figure 2.1

Interaction between Vitamin A Status, Infection and Susceptibility to further Infection



considerably longer than deficient rats in a conventional environment (Rogers et al, 1971). Experimental innoculation of vitamin A deficient chicks with Newcastle Disease Virus caused squamous metaplasia of nasal mucociliated epithelia, keratinization of bursal epithelia and depletion of mucosal lymphocyte populations compared with A-deficient, uninfected controls (Bang et al,1972). The authors emphasize that focal keratinization

of chick nasal mucosae occurred only in areas where NDV had destroyed the epithelium and suggest that vitamin A deprivation may prevent normal differentiation of residual basal cells. Such keratinization may also follow mechanical injury to epithelium (Wilhelm, 1954). Bang noted that the effect was organism specific - caused by NDV, but not influenza virus.

Such organism specificity may be important in the interaction between measles infection and vitamin A status. Measles is often quoted as a precursor of xerophthalmia (Oomen, 1969; Franken,1974; Inua,1983). The association may be so common that they may both occur together in'epidemic waves' (Oomen,1969), but is less common where vitamin A deficiency is not endemic (Inua,1983). Measles is associated with acute depression of serum retinol levels (Inua, 1983; Bhaskaram,1986). Children with post-measles lesions have lower plasma retinol than those without (Inua,1983), although Bhaskaram reported no association between type of eye lesion and plasma retinol.

A controversy centers on whether corneal lesions, often but not always present with infection in deprived populations, could be due either to the effects of measles virus or of vitamin A deficit (Bhaskaram, 1986). The argument against vitamin A involvement asks why, if vitamin A deficiency alone is responsible, blinding lesions do not occur as frequently in other childhood infections such as whooping cough, bronchopneumonia, chicken pox, etc. Moreover there is strong evidence of direct viral involvement in corneal lesions in measles (Inua,1983; Lancet,1987). It would be reasonable to expect both factors to be important, however. If marginal vitamin A deficiency prevents normal regeneration of such a virus-specific lesion, then an organism-specific interaction would occur.

Decreased tear production due to a vitamin A deficit may enhance virally initiated tissue breakdown and encourage necrosis due to secondary infection. The combined immunodepressant effects of protein-energy malnutrition, measles infection itself and marginal vitamin A deficiency may enhance the likelihood of such secondary infection. Thus an interaction between vitamin A deficiency and measles infection, rather than the presence of either, separately, may contribute to lesions.

The high mortality from measles in developing countries could be the result of a similar interaction (Dossitor et al.1977; Barclay et al. 1987). Superimposed vitamin A deficiency may hamper recovery from complications of measles, such as pneumonia, for similar reasons. This may increase mortality to a greater extent than when either vitamin A deficiency or measles infection is present alone. However the difficulty of demonstrating simple, first-order risk factor relationships for measles mortality has resulted in recent controversy and downplaying of the role of malnutrition in favour of less explanatory factors such as crowding and inadequate primary care (Anonymous 1983,1984).

2.4.2 Effect of Vitamin A Status on Infection

The form in which an interaction between vitamin A status and infection may be expressed, is determined by the stage in the infectious process which is affected. Thus a risk factor for an infectious disease can influence its outcome if it affects exposure, transmission, viral attachment, viral spread and the systemic response or recovery. Based on the evidence cited in section 2.2 and 2.3, vitamin A deficiency could be expected to affect all stages subsequent to transmission. Thus, in conditions of vitamin

A deprivation, an animal may be

a. more likely to acquire infection due to deficits in mucosal structure and function and in local immunity
b. infection is more likely to spread due to deficits in T-cell function, in macrophage and neutrophil activation
c. systemic infection is likely to last longer and be potentially lethal due to: delayed and reduced specific immune response, both humoral and cell-mediated (reduced numbers of T and B-cells, reduced rates of proliferation in response to antigenic stimulation, reduced antibody production)
d. pathology is likely to be more severe and recovery slower, due to effects on differentiation and reduced rates of proliferation of damaged tissue

An interaction could therefore be expressed in terms of incidence and duration of infection, mortality and long term sequelae.

Many early workers attempting to study the pathology of vitamin A deficiency were struck by the frequency of infection in their test animals and patients (Green and Mellanby, 1928; Chole, 1979; Bichler and Wieser, 1982). Green and Mellanby reported frequent infections of tongue, intestine (associated with particularly high mortality), kidney, bladder, prostate, nasal sinus, middle ear and lung in deficient rats compared with controls and coined the term 'anti-infective vitamin'. Bloch (1928), in selecting children admitted to hospital with either vitamin C or vitamin A deficiency, found 80% of vitamin A-deficient children with infections (pneumonia, bronchitis, otitis media, pyuria or pyoderma) compared with 30% of vitamin C-deficient children. McLaren (1965) found more infections in children admitted to hospital with xerophthalmia than with kwashiorkor

or marasmus alone. Scrimshaw (1968) reviewed a large number of studies which described circumstantial evidence of a relationship between vitamin A and resistance to infection.

However the tacit assumption of universal protection from infection amongst workers at the time, and the simplistic model implied in the term 'anti-infective vitamin' were soon undermined. Barenberg and Lewis (1932) reported that increasing the amount of vitamin A in the diet of infants receiving adequate diets did not reduce the number of respiratory infections. Likewise, halving the vitamin A content of milk did not increase the number of infections (total vitamin A intake not given). Lewis and Haig (1939) reported no increase in infections in infants on 1/12 normal dietary intake of vitamin A, but with normal dark adaptation responses. Disillusionment with the 'magic bullet' theory of vitamin A action was expressed as recently as 1979 by Hof and Wirsing. They found no difference in the immune response of vitamin A deficient and control mice in response to intravenous infection with Listeria monocytogenes, but found increased resistance to infection in normal mice given high doses of vitamin A. The authors state 'anti-infective vitamin does not hold absolutely true for vitamin A, although certain anti-infective properties cannot be denied'.

It can be predicted that any interaction between vitamin A status and susceptibility to infection is unlikely to be a simple, first order event because of the number and complexity of the multiple regulatory systems which govern both the immune response and the availability of vitamin A. Complexity of interaction can also be expected if the relationship is a non-linear one, for example if it operates only at low

levels of vitamin A status, or in the event of high frequency of infection. If these conditions were true, children on adequate diets or without recurrent infection would show no correlation between vitamin A status and respiratory morbidity. Thus anthropmetric indices of nutritional status are related to mortality risk in measles, only below certain thresholds (Nieburg and Dibley, 1986).

Similarly a universal protection model would not be appropriate for an interaction involving a multistage process where only one stage is involved, eg intraperitoneal innoculation of test organisms will not allow expression of an interaction if breakdown of mucosal barriers is the stage at which the interaction is operating.

A magic bullet theory will not fit if a factor operates at one stage, but is not normally rate-limiting for that stage. Thus less-than-optimal vitamin A availability may reduce T-cell mitogenic response, but sufficient reserve proliferative capacity may exist so that no difference is observed in outcome. Under these circustances, an effect may only be observed when a third factor comes into play which removes that reserve eg prior infection, measles, protein-energy malnutrition (Inua.1983), immuno-suppressive drugs, injury or metabolic stress.

The source and type of infecting organism would also be likely to add variability to an interaction between vitamin A status and susceptibility to infection. Thus Rogers and Bieri (1971) found naturally acquired multiple infection was lethal in vitamin A deficient germ-free rats, while oral innoculation of E.coli was benign.

Faced with an infinite series of variable combinations, the question of whether vitamin A status interacts significantly with susceptibility to infection is best asked for a defined group of people, with known vitamin A status and morbidity, so that the significance of such an interaction in terms of its impact on incidence and morbidity of the group can be assessed. Given the large body of evidence relating vitamin A status to immune function and infection resistance, the absence of studies in this area is remarkable. The only community studies assessing the impact of vitamin A status on infectious disease morbidity and mortality have been carried out by Alfred Sommer's group in Indonesia.

In a prospective study of 4,600 children in West Java, children between 3 months and 7 years of age were examined three monthly over a period of eighteen months (Sommer et al 1983a,b). Children were examined every three months for signs of ocular disease, general health status, height and weight and parents questioned about medical history during the interval. In an unusual method of analysis, the authors compared mortality during 3-month intervals considered by vitamin A status at the beginning of that interval They found higher rates of age specific mortality in ocular disease inititated periods then in those child periods where vitamin A status was 'normal' initially. Each child's mortality experience was thus counted more than once. Justification for this type of comparison was a high rate of night blindness and Bitot's spots which underwent spontaneous regression. A period initiated with eye symptoms was designated a vitamin A deficient period, and those without. designated vitamin A adequate. Mortality rates per 1000 child intervals were compared. The implicit assumption that the mortality experience of children recovered from eye lesions is the same as those in which lesions

had never occurred is questionable. Vitamin A deiciency develops over a long period of time. Children whose eye lesions regress are probably in a very similar deficiency state in the three months after remission. Likewise in the period before they appeared, there is a high probability of marginal vitamin A deficiency. Although this effect is conservative, it would be expected to cause a significant loss of discriminatory power in the study.

A second serious problem is the confounding effect of malnutrition. Sommer found no difference between normal and xerophthalmic child periods in weight for age. Some weight plateau and slowing of growth is an early effect of vitamin A deficiency. This, again suggests that his test and comparison groups were very similar with respect to their vitamin A status. The use of anthropometric indices as indicators of malnutrition, when they are also directly affected by vitamin A status seems pointless. This was not noted by Sommers critics, (Dibley et al, 1983) who suggested he use height for age, instead of weight for height to control for pem. Other indices exist which are independent of vitamin A status, for example serum albumin. However in areas where vitamin A deficiency is prevalent, the very close association with protein energy malnutrition may make assessing the effect of vitamin A deficiency separately, very difficult.

Despite the limitations of the study, namely that the difference in vitamin A status between the test and control child periods is probably minimal, and the effects of a potentially serious confounder have not been adequately controlled for, Sommer's data on respiratory illness in relation to ocular status is very interesting. The number of examinations in which upper respiratory illness was present varies from 7.9 per 100 child

examinations in the normal group, 11.2 in those with night blindness to 14.9 in those with night blindness + Bitots spot (Children with frank xerophthalmia and life threatening respiratory illness were excluded from the study). Thus increasing prevalence of mild respiratory illness was associated with increasing severity of vitamin A deficiency (or with malnutrition).

In a subsequent paper,(Sommer et al,1984) Sommer deals to some extent with these criticisms. He compares child intervals which were normal for ocular status at the beginning and end with those which were symptomatic at beginning and end and thus chooses comparison groups with more stable vitamin A status. Age specific prevalence rates of respiratory illness were significantly higher in all vitamin A deficient intervals except those in the over 5years age group. 'Relative risks' ranged from 1.9 to 2.4 (Since these figures are measures of relative point prevalence rather than incidence, relative risk may not be an appropriate term). It is of interest that the' relative risk' for diarrhoea was even greater (3.1 to 3.4) than that for respiratory infection. Among those children of adequate weight for length, more respiratory illness and diarrhoea was consistently found in the xeropthalmic groups (the same was true for children with weight for length <90%, however malnutrition is potentially greater in its effect as a confounder in this group, so less weight can be put on the comparison).

It is not possible to determine to what extent increased diarrhoeal and respiratory illness contributed to overall mortality in this study, because cause of death information is not available. However the increase in mortality with vitamin A deficiency seems relatively greater than that

in illness incidence for these diseases. Thus a 2 fold greater prevalence of respiratory illness and diarrhoea was associated with a 4 fold higher mortality amongst three year olds.

In a subsequent intervention study of vitamin A and preschool mortality, conducted by Sommer's group, (Sommer et al, 1986) 450 villages were randomly assigned to receive vitamin A supplementation (2 capsules, 20,000 iu) or to serve for one year as a control. The authors reported 49% overall lower mortality in the preschool age group, the difference expressed mainly in males. The study suffered a number of problems, however, most notably the lack of baseline mortality data. Moreover baseline morbidity data suggested that the control group contained sicker children, throwing doubt on the comparability of prospective illness experience of the two groups. An atypical pattern of age-specific mortality suggested that enumeration problems may have been significant in the control group (Gray, 1986; Costello, 1986).

In a third study (Barclay et al, 1987), 180 children who were admitted to hospital with measles, were randomly allocated to receive vitamin A (2 doses of 200,000 iu) or to receive standard treatment. Mortality was recorded over a period of one month following onset of rash., Groups were comparable for age, nutritional status, interval between rash onset and admisson. Of the 88 children who received vitamin A, 7% died, compared with 13% of the 92 who received standard treatment. This difference was not significant and was only observed in children under 2 years. It is unlikely that the power of the study was adequate to detect a difference in mortality, and the effects of supplementation on the incidence

of pneumonia, one of the complications of measles documented in the study, is not given.

The problems encountered by Sommer and his colleagues illustrate some of the difficulties of assessing vitamin A impact at the community level, and are possibly the main reason why, apart from his work , very little has been done.

Chapter 3

Measurement of Vitamin A Status: Analysis of Buccal Cells

3.0 Introduction

Plasma retinol is not a particularly useful measure of vitamin A status for a number of reasons. Its independence of dietary vitamin A intake once liver stores are adequate means it is a sensitive index of vitamin A status over only a limited range (marginal and clinical deficiency, <30ug/dl). While this range may be physiologically important from the point of view of clinical deficiency, there are indications that increasing vitamin A status above this level continues to affect function and disease resistance. Thus, increasing dietary intake of vitamin A reduces the risk of epithelial cancer in normally nourished populations (Ziegler et al, 1984,1986), while no clear relationship exists between serum retinol and risk of cancer (Friedman et al, 1986). Similarly, a relationship was found between dietary intake of vitamin A and dark adaptation in normally nourished Guatamalan children (plasma retinol >30ug/dl), while no correlation was found with serum values (Solomons et al, 1982). Increasing dietary intake is reflected in liver stores, in adequately nourished individuals, and assessment of liver stores may provide a better measure of vitamin A status. The relative dose response is a technique for assessing liver stores (Loerch et al 1979 , Flores et al. 1984). Liver reserves, themselves however, may be independent of the cellular availability of the vitamin (section 2.1.7). The release of vitamin A from liver is determined by factors which are independent of vitamin A stores (section 2.1.4). A direct measure of availability at the cellular level would therefore seem particularly desirable for vitamin A.

A number of studies have measured proteins which bind retinoids

intracellularly. These cellular retinoid binding proteins(CRBP) are present in a number of different species and in a wide range of different tissues (Chytil and Ong, 1984; Ong, 1985). However the exact function of CRBP and its relationship to cellular utilization of vitamin A is not known. Few studies have looked at tissue levels of retinol itself as a measure of vitamin A status. For the purposes of the study, an ideal measure would be cellular levels of retinol in respiratory tissue, or tissue closely related to it. The oral mucosa is continuous with the lining of the respiratory tract and displays similar sensitivity to vitamin A status. Deficiency causes the normally stratified squamous epithelium to keratinize (Baume et al, 1970). This effect can be reversed by topical application of vitamin A (Mill et al. 1982), and high vitamin A concentrations can cause mucous metaplasia in the tissue (Wong, 1975). Although it is not possible to quantitatively establish the relationship, it seems reasonable to predict that the buccal mucosa may reflect changes which occur in the respiratory mucosa with variable availability of vitamin A. Because of the non-invasiveness of the collection method, and the ease of obtaining large quantities of material, this tissue seems particularly suitable for epidemiological studies.

A method of sampling this tissue, and extraction of lipids was pioneered by McMurchie et al (1983,1984) who were interested in the fatty-acid composition of phospholipids. The cell collection and extraction procedures described by these workers were modified, a reference standard chosen, and new procedures for separation and detection by high performance liquid chromatography developed, based on those already in use for plasma retinol determinations. Because of the relevance of a-tocopherol to retinol stability and utilization, and because of the ease of

coextraction of this fat-soluble vitamin, cellular a-tocopherol levels were determined simultaneously.

This work took place after the first randomized, controlled trial of vitamin A supplementation and was sufficiently advanced for cheek cell collections to be incorporated into the second trial. It was undertaken in collaboration with the Department of Chemical Pathology at the Adelaide Children's Hospital, who carried out and were responsible for the development of all biochemical procedures. The analytical method was reported by Badcock et al, 1986. The stages in development of the method were as follows:

1. Analytical Method Development
    a. collection procedures
    b. extraction procedures
    c. separation and detection procedures
    d. reference standard development
    e. stability testing
2. Validity and Reliability Testing
3. Use in the Second Vitamin A supplementation trial.

The results of this work are described below.

3.1. Analytical Method Development.

3.1.1 Collection Procedures

The procedure which yielded most cells was vigorous rinsing of the mouth three or four times with a small amount of distilled water, prior to eating that day (mechanical scraping was avoided as it may induce bleeding). The procedure is outlined in instructions to participants in the Cheek Cell Study (appendix 3.1). Up to 4 to 8 mg wet weight cells could be collected from adults in this way, however, the yield from children was sometimes considerably less. The cell washings (approximately 20 ml) were collected in a light-protected vial containing butylated hydroxy toluene (BHT) to prevent oxidation, and kept at 5 C until transported to the laboratory for analysis. Sample were filtered through a sintered glass filter to remove any particles of food or debris (relatively few in fasting collections) and the filtrate centrifuged at 2000g for 10 minutes. The resulting pellet was washed by resuspending in isotonic saline and centrifuging twice more. Subsequently the pellet was either frozen at -20 C or resuspended in 2 ml of distilled water with successive vortexing and sonicating for 30 seconds for immediate analysis.

3.1.2 Extraction Procedures

Aliquots of 1.2 ml were taken for retinol analysis. To this 3 ml of 1-naphthol (100nmol/l in methanol) was added as internal standard and 1.5 ml of chloroform. After sonication (10s) and vortexing (60s) further water (1.5 ml) and chloroform (1.5 ml) were added to remove remaining

traces of water soluble material and the fat soluble phase removed and evaporated to dryness. Vitamin A standards of 0 to 50 pmol/mg protein were prepared by adding known amounts of all-trans retinol to the cell suspension.

Extraction for vitamin E determination was similar. To 0.4 ml aliquots of cell suspension, 0.4 ml of pyrogallol solution (50 mmol/l), an antioxidant, and 2 ml of tocol solution (internal standard) were added. Tubes were sonicated and vortexed (60s) as before and further water (1 ml) and chloroform (1 ml) added and the organic phase evaporated to dryness as before.

3.1.3 Separation and Detection

Retinol and a-tocopherol were subjected to high performance liquid chromatography on two separate columns. For retinol a silica gel column eluted with hexane-dioxane (92:8 v/v) was used, for a-tocopherol a reversed phase column eluted with methanol.

Fluorescence detection provided the sensitivity required to detect the low cellular vitamin A concentration. The excitation wavelength was 326 nm for retinol, 295 nm for a-tocopherol while emitted light was detected at 470 nm for retinol and 330 nm for a-tocopherol. Details of columns and elution times have been reported by Badcock et al (1986). Peak height ratios of vitamin standard to internal standard were used to calculate sample retinol values. The detection limit, defined as a signal

twice the noise level, was approximately 0.5 pmol retinol per mg protein and 5 pmol a-tocopherol per mg protein.

3.1.4 Choice of Reference Standard

A reference standard was needed to quantify the amount of material being analysed. For this purpose wet weight, dry weight, cell count, phospholipid, protein and DNA/RNA were considered. Practical difficulties were encountered in weighing the very small amounts of material and protein proved to be a more reproducible measure of biomass (coefficient of variation-3%). Protein was correlated with dry weight by a factor of 0.8 (n-18).

3.1.5 Stability

Stability was an important consideration given the susceptibility of retinol to oxidation and destruction by light. The cheek cell pellet was stable when stored for up to 10 weeks at -20 C. Stability during collection and analysis, determined by recovery of added vitamins A and E, was greater than 89% for both vitamins. It was necessary to add pyrogallol during extraction of vitamin E to achieve this level of recovery.

3.2. Reliability and Validity Testing

3.2.1 Background

The question of validity testing is an interesting one. To what extent buccal retinol measured in this way represents true availability of vitamin A within the cell or within cells of the respiratory tract i.e. internal validity cannot be determined, as there is no independent measure of this. The correlation of buccal retinol with plasma retinol, for example, would not be expected to be high if buccal retinol is a true measure of tissue level availability, since plasma retinol is such a measure over only a limited range. It could well be that within that range (<30ug/dl), some correlation would be expected, however.

The face validity of the measure is high i.e. the quantity of retinol within the cell could be deemed a good estimate of its availability within the cell. However this depends on whether the amount present within cell at any one time, fluctuates widely . This 'biological constancy' is thus fundamental to the validity of the method.

The reliability of the method refers to the repeatability of the estimate on different occasions , and thus to the variability inherent in the analytical procedure. This is comprised of error in the collection procedure ('sampling variation') as well as error occurring during extraction and detection procedures.

An analysis of validity and reliability of the method would therefore

involve assessment of the error derived from variation within one person over time (biological constancy) and on different occasions at any one time (reliability). The size of this relative to between person variability would give an estimate of the usefulness of the method as a measure of individual vitamin A status.

3.2.2 Method

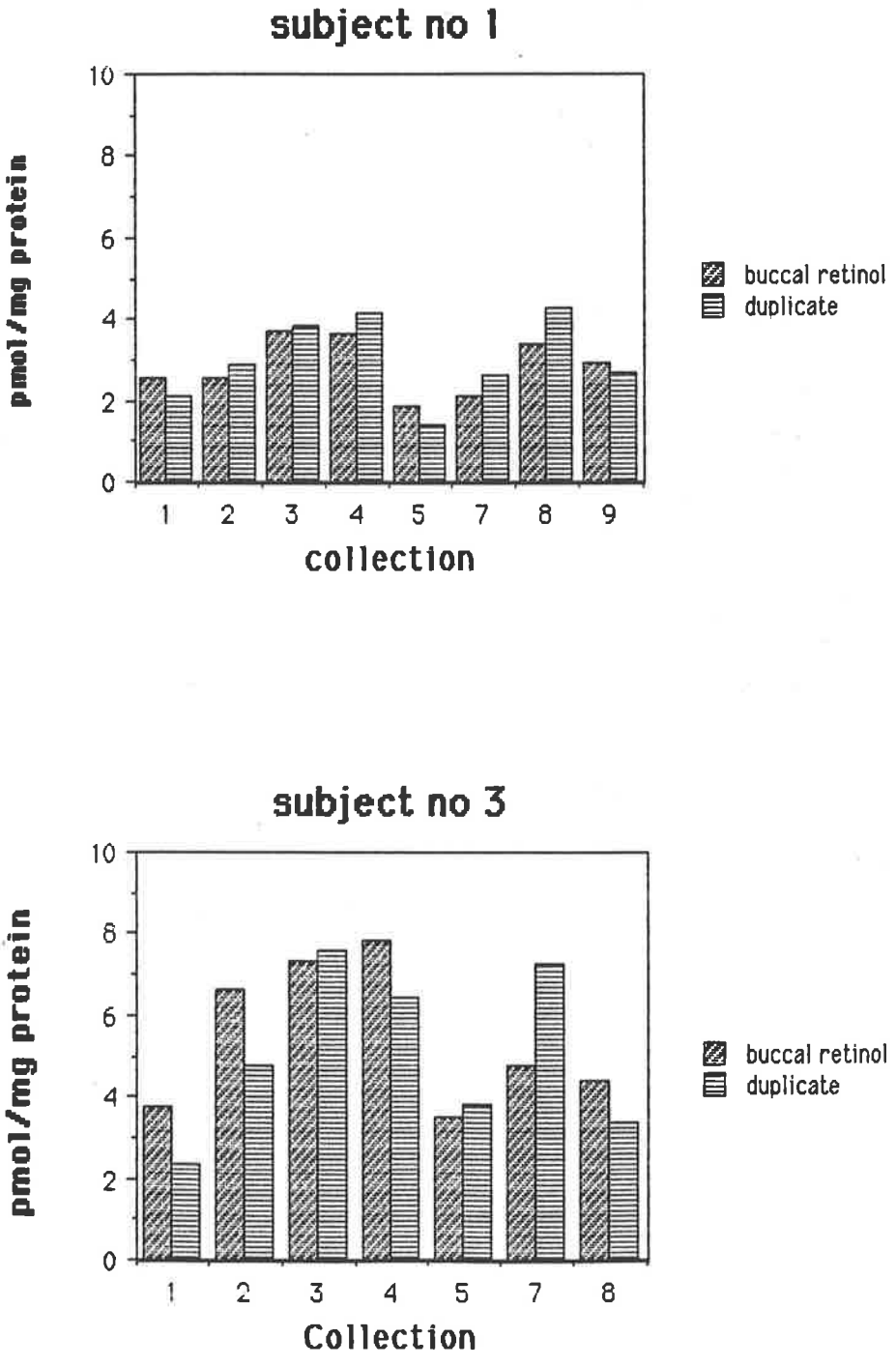
Nine repeated collections were made at two day intervals on 11 subjects. Each collection was analysed in duplicate. This design allowed measurement of three components of variability, namely analytical error, error due to changes over time within individuals and error due to between person variability.

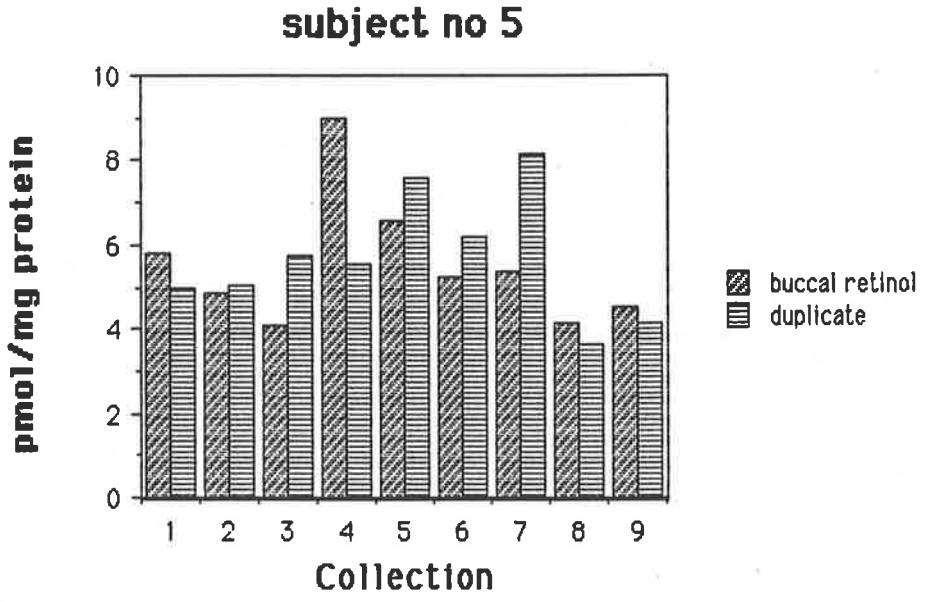
Subjects were adults between the ages of 20 and 55 years, who volunteered to take part in the study. Instructions to participants are shown in appendix 3.1 . Mouth washings were collected in a darkened plastic screw-cap jar upon rising in the morning , immediately refrigerated, and transported in cool boxes to a collection point, where transfer was arranged to the laboratory. Samples were filtered, spun and stored as the washed pellet at -20o C and analyses performed as described above.

3.2.3 Results

A total of 159 plasma retinol determinations were made on 11 subjects with an overall mean of 5.4 pmol/mg protein. Individual means ranged

Figure 3.1 Buccal Retinol Values of Four Subjects in the Reliability/Validity Study

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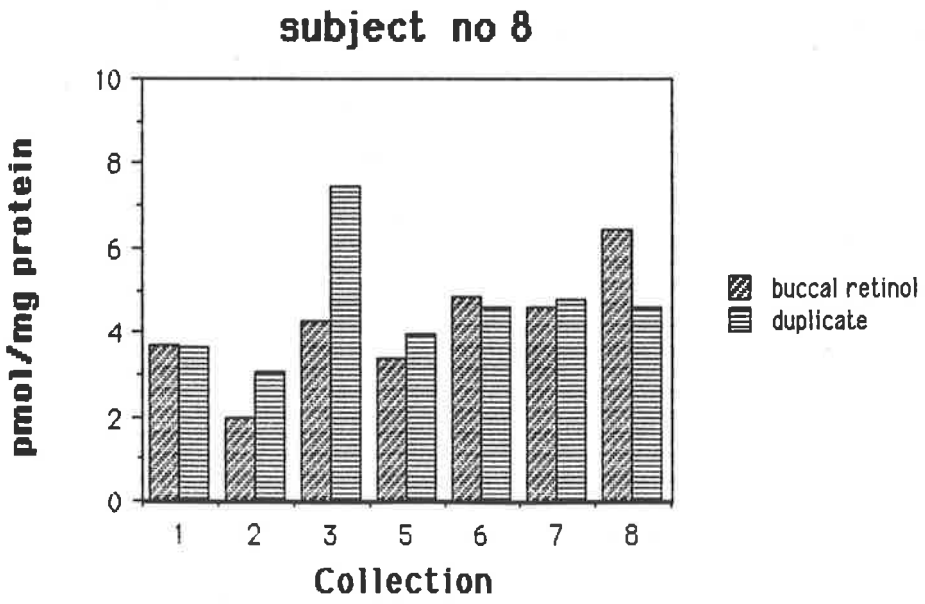
pmol/mg protein
10
8
6
4
2
0
1 2 3 4 5 6 7 8 9
Collection
buccal retinol
duplicate


subject no 8
pmol/mg protein
10
8
6
4
2
0
1 2 3 5 6 7 8
Collection
buccal retinol
duplicate

from 2.4 - 11.7 pmol/mg protein. Examples of individual distributions are shown in figure 3.1. A nested analysis of variance was used to partition variability . Results are shown in table3.1.

Table 3.1
Analysis of Variance in 159 Determinations of Buccal Cell Retinol

Source	Degrees of Freedom	Sums of Squares	Mean Square	F
Between Subjects	10	1611	161.1	35.5**
Between collections	73	332	4.5	3.16**
Error	76	109	1.4	
Total	159			

"Between subjects" refers to the variability due to the 11 different subjects tested. "Between collections" refers to intra-individual variability over the 9 different occasions on which collections were made and "error" refers to the remaining variability which corresponds to measurement error. Both subject (interindividual variability) and collection (intraindividual) effects are statistically significant, that is to say variability from these sources is a significant contributor to overall variability. Application of a components of variance model allows comparison of the magnitude of their contribution to the overall variance. The sources of variability in the model can be

related in the following way:

$$Yijk = U + Si + Cj(i) + Ek(ij)$$

where Y is the observation on the ith subject, jth collection and kth duplicated. S is the subject effect, C is the collection effect and E is the analytical error as measured by duplicates. (The model assumes both subjects and collections are random effects i.e subjects are chosen at random from a much larger population of subjects and the collections within those subjects are a random sample of all possible collections). Variance calculated in this way is shown in table 3.2.

Table 3.2
Components of variance in 159 Determinations of Buccal Cell Retinol

Source	Variance	%
Subjects	8.7	74.5
Collections	1.6	13.3
Error	1.4	12.2
	11.7	100

It can be seen that 74.5% of the variability in the 159 measurements can be attributed to between subject variability, while only 13% of the variability is due to variation over time with different collections, 12% is due to variability in the analytical method. The small size of the measurement error, compared with the between person variation suggests

that this may be a useful estimate of vitamin A status.

Sources of this measurement variability (25.5% in total) not already mentioned. include loss of cell contents during the collection process , instability of retinol in the cell suspension and loss of material during filtration and extraction . These sources of error are probably not independent of sample size. At very low yields, the opportunities for loss of material may be much greater. This increased source of error at low yields may be a problem in small children, where due to difficulties in understanding the procedure (eg swallowing the washings), yields may be quite low.

3.3 Vitamin A Supplementation Trial

An opportunity for examining the distribution of buccal cells in a group of children was provided by the second supplementation trial. Buccal collections were made on children 4 years and above at the beginning of the study, and 12 months later at its conclusion. The method of collection was the same as used in adults, but smaller volumes of water were used, and frequently the first rinse was ommitted. With children 4 years or younger, cooperation was a problem, because of difficulty understanding what was required. The washing procedure was demonstrated. and the child invited to imitate, but frequently, the temptation to swallow once water was held in the mouth, was just too great. This meant that yield was low, particularly at the initial collection, and provided quantities of material which were either inadequate or at the limits of sensitivity of the

method. The results of these collections are described in the following sections.

3.3.1 Distribution of Buccal Retinol.

Buccal retinol determinations were made on 135 children at the beginning of the study and 100 children at the end. Mean, standard deviation and the 25th percentiles of the distribution are shown in table 3 .

Table 3.3
Distribution of Buccal Retinol and a-Tocopherol in Children in the Second Vitamin A Supplementation Trial.

Var-iable	N	Mean (pg/mg prot)	SD	min.	Percentiles 25%	50%	75%	Max
Buccal A initial	135	161	135	6.9	61	122	227	705
Buccal A final	100	188	167	17.2	65	125	254	730
Buccal E initial	121	6.8	6.1	0.21	2.5	5.5	7.9	33.2
Buccal E final	141	4.9	3.2	0.54	2.5	4.3	6.5	17.7

Mean buccal retinol was similar to that in adults (overall mean 154pg/mg protein), however the variability was considerably greater in the sample of children (CV 88% compared to 66 % in adults and children, respectively),

and was reflected in the wide range of values encountered (maximum 452 in adults, 705 in children). This variation may be related to larger measurement error in children than observed in adults, or it may reflect the difference in size of population measured (N-11 in adults, N-100 in children). Alternatively, it may represent a true difference in distribution of buccal retinol between adults and children. The distribution is skewed to the right, slightly (table 3.3) and somewhat truncated at the lower end.

3.3.2 Correlation Between Initial and Final Measurements

An assessment of the repeatability of measurements is provided, to some extent by the correlation between initial and final buccal determinations. If buccal vitamin A is a stable index of vitamin A status, some long term consistency between determinations would be expected. Table 3.4 shows correlations between initial and final determinations for both vitamin A and vitamin E. Correlations in sub-groups of the population, based on initial plasma retinol values are also shown, because these subgroups are important in the comparison of buccal and plasma retinol described in the following section.

Table 3.4
Association between Initial and Final Determinations of Buccal Vitamin A and Vitamin E (Spearmann's Correlation Coefficients)

Association	Total Population	Plasma A < 40 ug/dl	Plasma A < 30 ug/dl
Buccal Vitamin A (N, significance)	0.24* (77,0.02)	0.39** (36,0.009)	0.38 (13,0.10)
Buccal Vitamin E (N, significance)	0.20* (96,0.02)	0.31* (39,0.03)	0.26 (130.20)

A weak, but significant correlation between initial and final measurements was found for both vitamin A and E, in the total population and in the subgroup with plasma retinol < 40 ug/dl.

3.3.3 Association between Buccal Vitamin A and Plasma Vitamin A

No correlation was anticipated between buccal vitamin A and plasma retinol in the total population because in populations with adequate vitamin A status, plasma retinol is independent of dietary intake (section 3.0). However plasma retinol levels in the second trial were lower than the first, and included a group of children who could be considered at risk of marginal deficiency (chapter 5). If buccal retinol is to be considered an

index of vitamin A status. then some correlation with plasma retinol would be expected in this group (those with plasma retinol < 40ug/dl, section 5.4.6). Spearmann's Correlation coefficients for the variables in the total population and in the two subgroups are shown in table 3.5.

Table 3.5
Association between Buccal Vitamin A and Blood Vitamin A
(Spearmann's Correlation Coefficients)

	Total Population	Plasma A <40 ug/dl	Plasma A <30 ug/dl
Initial (N, Significance)	-0.01 (112,0.43)	-0.03 (61,0.39)	-0.07 (20,0.38)
Final (N, Significance)	0.13 (87.0.11)	0.27* (43,0.041)	0.49* (16,0.026)

As expected, no statistically significant correlation was observed in the overall population. However if groups of children with plasma retinol < 40 ug/dl are considered separately, the correlation was stronger and significant ($r=0.49, n=16, p<0.05$). This was observed in the final collections, but not in the initial ones. It is possible that our experience with the technique and ability to standardise procedures and thus minimize variability, and the greater quantity of material gathered from older children improved results during the second set of buccal collections, and that this therefore gives a better representation of the correlation. (Vitamin A supplementation is unlikely to contribute to this effect since the correlations with final plasma retinol were the same in treatment and

placebo groups).

3.3.4 Association between Buccal Vitamin A and Dietary Vitamin A

Dietary intake was measured in two ways - a frequency questionnaire at the beginning and end of the study, and a three day diet diary half way through the follow-up period. Dietary intake of vitamin A estimated in these ways was then combined with the treatment variable to give total intake of vitamin A including the supplementary dose. Correlations were examined within sub-groups as before. Results are shown in table 3.6.

Table 3.6
Correlation Coefficients for the
Association between Buccal Vitamin A and Dietary Vitamin A

Dietary Vitamin A Variable	Total Population	Plasma A <40 ug/dl	Plasma A <30ug/dl
Frequency : initial	-0.08	-0.19	-0.79**
(N, significance)	(98,0.20)	(45,0.10)	(15,0.00)
Frequency: final	0.06	0.13	-0.35
(N,significance)	(84,0.29)	(41,0.21)	(17,0.10)
Diary: correlation	-0.01	0.03	-0.14
with inital buccal	(101,0.44)	(46,0.43)	(15,0.30)
Diary: correlation	-0.01	0.05	-0.67**
with final buccal	(86,0.46)	(42,0.38)	(17,0.002)

No significant associations were seen between dietary intake of vitamin A and buccal retinol in the total population or in the subgroup with plasma retinol <40ug/dl. In the sub-group with lowest plasma retinol, a significant negative correlation was seen between initial buccal retinol and initial frequency dietary intake, and final buccal and the dietary estimate of vitamin A intake. Other associations were also negative, but not significantly so. Examination of dietary correlations with plasma retinol in this group did not show the same consistency and strength of correlation. It is difficult to explain negative correlations with diet unless it could be postulated that while dietary intakes increase with age in this group, tissue levels decline. This would occur in the event of difficulties of utilization of vitamin A (uptake, mobilization and transport) which are postulated in

connection with this group in chapter 6.

Mean buccal retinol in supplemented and unsupplemented groups is shown in table 3.7. Mean buccal retinol did not change in the supplemented group. This is consistent with the lack of association with dietary vitamin A in the total population.

Table 3.7
Mean Buccal Retinol in Supplemented and Unsupplemented Groups (pg/g protein)

	Placebo			Supplemented		
	Mean	SD	N	Mean	SD	N
Initial	168	160	44	161	161	57
Final	210	194	37	172	153	49

Lack of an association with dietary intake is surprising. Some correlation with diet would be expected if dietary intake affects cellular levels of retinol in this population. Independence of cellular vitamin A from dietary intake, may however reflect the special nature of this population (chapter 5) .Plasma retinol also did not increase in response to supplementation in this group, even at low initial plasma vitamin A levels and the question is raised whether the population is responsive in any way to dietary intake of vitamin A (chapter 5).

3.4 Conclusions

Buccal retinol determinations during the second vitamin A supplementation trial established that:

1. There is a correlation between measurements taken one year apart.
2. Amongst children with low levels of plasma retinol. there is evidence that buccal retinol is correlated with plasma retinol.
3. In this group of children (chosen on the basis of hospitalization for RSV positive bronchiolitis in infancy), buccal retinol levels are not correlated with dietary intake.

Further work on this method should look at the relationship between buccal retinol and functional measures of vitamin A status over a broad range including levels of clinical deficiency. Normal ranges for buccal retinol, relating to functional measures of vitamin A status, could then be developed, and the sensitivity and specificty of the measure as a test of adequacy of vitamin A status, determined.

Retinyl palmitate, the storage form of vitamin A, frequently exists in higher quantities in tissue, and measurement of vitamin A in this form may provide and index which has a lower measurement error. The existence of significant between person variability relative to measurement error, the non-invasiveness of the test, and the ease with which it can be performed in primary age children and above, suggest that buccal cell vitamin A assessment is worth further development as an epidemiological tool.

Chapter 4

The Pilot Study

4.1. Introduction

The pilot study was conducted to investigate three key relationships within the context of vitamin A status and respiratory proneness. These were:

1. The relationship between dietary vitamin A and the number of respiratory events.
2. The relationship between blood vitamin A and the number of respiratory events.
3. The relationship between dietary and blood vitamin A.

An opportunity to do this was provided by a study, at the time underway in the Department of Community Medicine, in which respiratory symptoms were being measured prospectively over a three year period (Vaccine study). This was a trial of a pneumococcal vaccine in pre-school age children and involved sampling blood to determine antibody levels and a questionnaire on respiratory history of participants. It is discussed further in section 4.3.1. A cross-sectional survey was therefore possible linking the number of respiratory events with the level of vitamin A determined from blood samples and dietary vitamin A intake determined by interview. The association described above was consequently examined using Spearmann's Rank Order Correlation Coefficients.

4.2 Method

Questionnaires requesting information on the number of respiratory episodes during the preceding 12 months were collected for all children at the beginning of the vaccine study. A random sample of 49 1-5 year old children was chosen from the vaccine study . Serum vitamin A determinations were carried out by the Institute of Medical and Veterinary Science using a fluorometric method, on blood samples also collected as part of the trial. An estimate of dietary intake of vitamin A was obtained from a diet frequency questionnaire, administered to parents at interview. This requested frequency of consumption of 21 vitamin A- rich foods. in designated serving sizes on a weekly basis.The foods included were liver, spinach, carrots, tomato, broccoli, brussels sprouts, lettuce, celery, orange, bananas, apricots, peaches, pumpkin, beans, peas, milk, eggs, butter, cheese, cream, ice cream. Dietary vitamin A was expressed as daily intake of retinoids (preformed vitamin A), ß-carotene (provitamin A) and total number of vegetable serves per day (variable ALLVEG).

4.3 Results

Mean values of dietary and serum vitamin A are shown in table 4.1.
Results showed that:

1. Vitamin A intake was divided approximately equally between sources of preformed vitamin A (retinoids) and provitamin A (carotenoids).
2. Dietary intake was high and well above the recommended daily allowance, even when based on the restricted food list chosen.

3. Mean serum vitamin A levels were well above levels at which symptoms of deficiency are seen.

4. Dietary intake was weakly, but significantly associated with serum vitamin A in the case of total vegetable intake (r=0.27, n=45,p<0.05; ß-carotene intake r=0.23, n=45, p>0.05), but not with retinol intake.

5. Results of simple correlations between respiratory episodes, dietary and serum vitamin A are shown in table 4.2. Associations were found between dietary and serum vitamin A and episodes of respiratory illness.

Table 4.1
Mean Values of Diet and Serum Vitamin A In a Cross-Sectional Survey of 49 Preschoolers.

Variable	Mean	Standard Deviation
Dietary retinol (ug re/day)	405	177
Dietary ß-carotene (ug re/day)	450	331
Total Dietary Vitamin A (ug re/day)	876	405
Serum vitamin A (ug /100ml)	67.9	18.7
Serum carotenoids (ug /100ml)	105	58

Dietary vitamin A was not correlated with respiratory episodes in the overall population. However inspection of a scatterplot of the bivariate distribution showed an apparent curvilinear relationship. A negative correlation occurred above 5 respiratory bouts in the previous 12 months (respbouts), a positive correlation below this level. While neither was

significant at the 5% level, the same curvilinear relationship was evident between serum vitamin A and number of respiratory events. This time the negative correlation above 5 respiratory bouts was stronger and significant ($r=0.43$, $p<.05$). The correlation below 5 episodes was not as strong, but significant also. Thus both dietary and serum vitamin A were related to respiratory episodes in a similar way. In children with frequent respiratory episodes, the relationship was an inverse one.

Table 4.2 Association between Respiratory Episodes and Dietary and Serum Vitamin A (Pearson's Correlation Coefficients)

	Allveg	Total Dietary A	Dietary B Carotene	Dietary Retinoids	Serum Vitamin A
All cases N=47	0.07	0.18	0.09	0.14	-0.05
Respbout>5 N=33	0.31	-0.18	-0.29	0.14	-0.43*
Respbouts<5 N=18	0.21	0.20	0.22	0.04	0.31*

* $p<0.05$

4.4 Discussion and Conclusions

The pilot study illustrated a number of problems in methodology and study design which were particularly relevant to vitamin A and shaped thinking for the remainder of the project.

a. Measurement of blood vitamin A

Vitamin A exists in three major forms in blood: ß-carotene, retinyl esters and protein-bound retinol. Both ß-carotene and esterified retinol are post-absorptive forms and their levels fluctuate diurnally, depending on intake. Protein-bound retinol is much more constant, being homeostatically maintained if liver stores are adequate. It represents vitamin A status when the latter is marginal, and therefore is the preferred form of vitamin A in blood to measure. The fluorometric method of vitamin A determination used in the pilot study, did not distinguish between post-absorptive and protein-bound forms of the vitamin. Since blood samples were non-fasting, this may explain why mean levels were quite high (68ug/dl). It draws attention to the importance of measuring only the protein bound form of vitamin A in non-fasting blood samples, and a method of doing this using high-performance liquid chromatography was subsequently developed by the Chemical Pathology Department of the Adelaide Children's Hospital.

b. Study Design

A number of problems with cross-sectional studies are particularly relevant to vitamin A. The difficulty of distinguishing cause and effect is important because respiratory illness itself can affect vitamin A status in many different ways. Thus serum vitamin A drops as a result of infection, and frequent infection is likely to reduce dietary intake and the efficiency with which vitamin A in the diet is used (section 2.2.2). These factors would be likely to cause an inverse association between vitamin A status and episodes of respiratory illness in a cross-sectional study. A positive association would be caused if parents concerned about perceived frequent illness offer more fruit and vegetables, or merely report more. A combination of these factors could cause a curvilinear relationship between vitamin A status and

episodes of respiratory illness, or, alternatively such a relationship could represent the partial obscuring of an underlying causal relationship between low vitamin A status and susceptibility to respiratory illness. The possibility of many different factors causing associations of the type seen in the pilot study meant that an alternative design was needed.

This thesis is particularly concerned with the effect of vitamin A status on susceptibility to respiratory illness, rather than the reverse relationship. Causal direction in an association can only be demonstrated by a design which changes the independent variable (in this case, vitamin A status) and measures the effect on the dependent variable (episodes of respiratory illness). Oral supplementation is the most direct way to achieve a change in vitamin A status, and a double blind, randomized trial is a design which eliminates bias as much as possible in the assessment of outcome. This type of study design was therefore used during the remainder of the project.

The pilot study gives preliminary evidence of an inverse correlation between dietary and serum vitamin A and episodes of respiratory illness in children with frequent respiratory infections. This group of children is of particular interest because of the possibility of a cyclical interaction between vitamin A status and susceptibility to infection in this group (section 2.4) - low vitamin A status contributing to frequent respiratory illness, and frequent infections, in turn, depleting vitamin A stores. Such an interaction would be particularly important in countries where vitamin A is endemic and at ages where respiratory infections are at their peak (both vitamin A deficiency and acute respiratory infections are most prevalent during the preschool years). This group of children (later termed 'respiratory prone' and defined in other ways) therefore became the focus of further investigations.

Chapter 5

Vitamin A Status and Respiratory Illness in Respiratory Prone Children in the Community

5.1 Introduction

The underlying hypothesis of interest to the thesis is that vitamin A status is causally related to respiratory morbidity. As discussed in chapter 4, such a directional hypothesis can only be investigated with an intervention study design: that is, one in which vitamin A status is changed, and the effect observed on respiratory morbidity. In this case the intervention chosen was oral supplementation with vitamin A. The specific hypothesis investigated, therefore, is whether changing vitamin A status by supplementation can reduce respiratory morbidity. It was proposed to carry out an intervention trial in which a group of children in the community who had experienced frequent episodes of respiratory infections during the period immediately preceding the trial were given a dietary supplement of vitamin A. Respiratory symptoms would be monitored over a 12 month period and the number of symptom episodes compared with those in an equivalent group of children, given an identical supplement, but which did not contain vitamin A. Both participants in the trial and investigators would be blind to which children received or did not receive the vitamin.

Whether the specific hypothesis is a test of the underlying hypothesis is determined by whether vitamin A supplementation, at the level chosen, can change vitamin A status. The level of supplementation chosen was 450ug retinol equivalents per day, the Recommended Daily Allowance (Food and Nutrition Board, 1980). The dose level chosen was conservative for two reasons: high dietary intake, approximately 10 times this figure, is toxic (section 5.3.3.3), and it was necessary to leave an adequate safety margin to allow for dietary fluctuations. The second consideration was that the underlying hypothesis was concerned with variability in vitamin A status.

normally encountered in the community. Thus a change on a dietary, rather than pharmacological scale, was called for.

Other factors of importance in the study were the measurement of only the protein-bound form of the vitamin in blood, thus eliminating variation due to post-absorptive fluctuations in retinyl esters, and the measurement of family history and environmental factors which are known risk factors for respiratory illness. The latter was necessary, not only to gain some idea of how significant the dietary contribution would be, but also to control for potential confounding factors.

5.2 Sample Size Determination

In order to estimate parameters necessary for sample size determination, cases in the upper one-third of respiratory symptoms frequency for the first six months of diaries collected as part of the vaccine trial were examined. Mean episodes (EPSARI) and days (DAYSRSP) of respiratory symptoms and days of cough (COFDAYS) in this group are shown in table 5.1. The number of subjects required to detect a difference of 20 % (type I error=0.05, type II error=0.80) is given.

Table 5.1
Sample Size Needed to Detect a 20% Difference with 80% Power (2-tailed test)

	Mean	SD	N (in each group)
EPSARI (>3)	5.54	2.0	52
DAYSRSP (>24)	44.9	22.5	98
COFDAYS	21.0	23.7	499

It can be seen that a total study population size of 200 would be adequate to detect a difference in the variables episodes and days of acute respiratory illness, but not the symptom variable, days of cough. A minimum of 100 would be necessary to detect a difference in episodes of respiratory symptoms only. It was decided to aim for a population size of 200.

5.3 Method

5.3.1 The Study Population.

The study population was drawn from children who were already participating in a three-year randomized, controlled trial of a polyvalent pneumococcal vaccine (Vaccine trial) directed by Dr R.M. Douglas in the Department of Community Medicine, University of Adelaide. Subjects in the vaccine trial, initiated in 1980, were drawn from three group general practices located at Ingle Farm and St Agnes Health Centres in the north-eastern suburbs of Adelaide and included 1273 children between the ages of six and 54 months at the time of entry. The trial had been in progress for 12 months when the vitamin A study was initiated . Subsequent analysis of the trial showed no consistent evidence of benefit to vaccine recipients for any period of the trial (Douglas et al, 1983; Douglas and Miles, 1984).

5.3.2 Selection of Subjects and Organization of Procedures

Subject recruitment for the vitamin A trial began in August 1981 with a circular to all participants in the vaccine trial (Appendix 5.1). All children

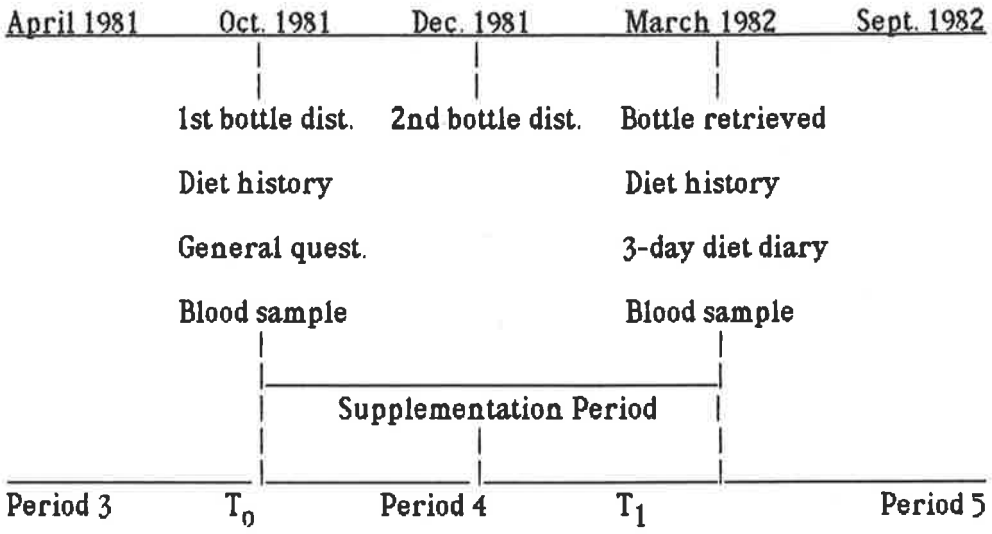
1-4 years of age who had registered on symptom diaries as having more than 15 days of cough or three or more separate episodes of respiratory symptoms in the past three months were eligible for inclusion in the study. Participants in the study who responded to the newsletter were visited by a nurse who further explained the study and distributed an information sheet (Appendix 5.2) and informed consent form (Appendix 5.3). A questionnaire (Appendix 5.5), designed to obtain nutritional medical history, family history, home environment and sociological information, was explained and left with parents, who were asked to bring the completed form to the first examination.

Two extensive interview-examinations with parents of children were conducted by the author, one at the beginning (in September, 1981) and one at the end of the supplementation period (March, 1982). During these visits (at Ingle Farm and St Agnes Health Centres), children were weighed, height was measured, fingerprick blood samples were taken by the nurse, and the diet/general questionnaire was reviewed with the parent .

Supplement bottles were distributed at the initial interview and at an additional short interview in mid December. At this interview, parents were questioned about the general health of the child. A summary of procedures is shown in Fig. 5.1. After each 6 month period, the project staff for the vaccine trial distributed and collected respiratory symptom diaries , and maintained a vigorous telephone follow-up to ensure return of the latter. Coding of respiratory diaries for the variables, episodes and days of respiratory symptoms was carried out by the field director (as part of the vaccine trial). All other coding and analysis of respiratory diaries, general questionnaires and diet diaries was carried out by the author.

Figure 5.1

Summary of Procedures



5.3.3 Vitamin A Supplementation Procedures

5.3.3.1 The Supplement

The vitamin A supplement and placebo were prepared by Faulding & Co., Thebarton, SA. Retinyl palmitate was dissolved in propylene glycol and made up to strength with a glucose syrup base to which raspberry essence had been added to improve palatability. No preservative was added, to maintain simplicity of formulation in case of side effects. Some problems with crystallization of sugar occurred towards the end of the storage period, however this was found not to affect the content of vitamin A by the manufacturer. The placebo was identically labelled and bottled, and consisted of syrup and raspberry essence. No difference in taste could be detected.

5.3.3.2 Dose Regimen

The dosage regimen was designed to delivery a mean daily dose of 450 ug retinol equivalents (the recommended daily allowance for this age group - Food and Nutrition Board, 1980) to recipients of the active preparation. This was achieved with a three times weekly dose of 2.5 ml of syrup. The bottle contained 97.5 ml, and thus was expected to last three months. Only two bottle distributions were therefore required for the full six-month supplementation period. Some fall-off in activity was expected during the period of the shelf life of the supplement, and the initial dose was formulated at 491 ug/ml retinol equivalents (re) to allow for this.

5.3.3.3 Toxicity

Toxic levels of vitamin A for infants have been quoted as 5500 ugre/day over a period of three months (Persson et al, 1965). The American Academy of Pediatricians has advised against use of preparations containing more than 1800 ugre/dose, however, while the Canadian Pediatric Society quotes 750 ugre/day as the recommended dose (Fomon, 1974). The level of dosage in this study was thus well below these maxima. A check on reactions to the supplement was made during the third interview when parents were asked the question "Has your child been well since taking this medicine?" Replies were recorded and compared with symptoms of hypervitaminosis A as follows:

Acute: transient hydrocephalus and vomiting;

Chronic: fatigue, malaise, lethargy, abdominal pain, bone or joint pain, severe throbbing headache, insomnia, restlessness, emotional lability, night sweats, constipation, scaly, rough skin, peripheral oedema, brittle nails.

5.3.4 Randomization Procedures

Vitamin A supplement and placebo bottles were randomized in the following way. Identically labelled supplement and placebo bottles were received from the manufacturer in two separate boxes. A person who was unconnected with the study was assigned to number the bottles. Each box was arbitrarily assigned to an odd or even category and a table of random numbers consulted sequentially. Bottles, taken in order from the boxes according to whether the random number was odd or even, were numbered serially from one to 147 and the source box recorded. The randomized bottles were then distributed sequentially to children coming to their initial interview.

5.3.5 Blood Procedures

5.3.5.1 Finger Prick Samples

0.2 ml of blood was collected by finger prick method in heparinized tubes. Samples were placed in a light tight container at $5^{o}C$. They were subsequently (within six hours) transferred to the Adelaide Children's Hospital where they were centrifuged, and the plasma stored at $-20^{o}C$.

5.3.5.2 Biochemical Analyses

All biochemical analyses were carried out by the Department of Chemical Pathology, Adelaide Children's Hospital. Plasma retinol and a-tocopherol were determined by high performance liquid chromatography using a method based on that of Bieri (1979). Plasma samples were prepared by heptane extraction and injected into a reverse phase column: the analytical column was a pre-packed 250 x 4.6 (i.d.) mm Ultrasphere ODS, average particle size 5um and the guard column was 50 x 4.6 (i.d.) mm dry packed with Ultrasphere ODS 20um (Beckman Instruments Inc.). All-trans retinyl acetate and d-a-tocopheryl acetate were used as internal standards. Plasma samples as small as 25ul were measured using this method. Use of an Altex 165 variable wavelength detector allowed monitoring of a-tocopherol and retinol at the wavelength at which their absorption is maximum (292 mm and 326 mm respectively), thus reducing the volume of plasma required.

A minimum of 25ul of plasma for ß-carotene determination was extracted into heptane to which an equivalent volume of 5-chloro-2 (methylamino) benzophenone was added. ß-carotene was detected at 450 n.m. The samples were injected onto an Ultrasphere Si 250 x 4.6 mm analytical column with guard Ultrasphere Si pre-column and eluted isocratically with hexane-dioxane (92:8, v/v) at a constant flow rate of 1.5ml/min.

In order to determine whether vitamins A and E were stable during the conditions of storage and transport in the study, several determinations were made in plasma under the following conditions of storage:

1. 24 hours at room temperature in the dark.
2. 1, 2, 4, 5, 8 days at 4^{O}C in dark amber vial.
3. Monthly for 6 months at -20^{O}C in dark amber vial.

Retinol and a-tocopherol were stable under all these conditions. Several determinations were also made on the vitamin A syrup supplement stored at 4^{O}C in an amber bottle over six months. Vitamin A content declined by 8% and 11% over this period in the two samples measured.

5.3.6 Measurement of Respiratory Symptoms

Symptoms of acute respiratory illness were recorded for the vaccine trial using a diary method based on that of Calabrese (1980). Diaries comprised six sheets on each of which the presence or absence of six symptom types could be recorded by parents daily, for a period of one month (appendix 5.7). Information on visits to general practitioners was recorded by the parent in a separate box in the diaryand doctors who saw the child completed a special sticker which was included in the child's medical case-notes (appendix 5.9).

Six-monthly summaries of diary and sticker information were prepared by nurses for the vaccine trial according to the coding schedule outlined in appendix 5.10. The variables of interest in the vitamin A trial were equivalent to those used in the vaccine trial, but were summed separately because they covered different time periods from those used in the vaccine trial. They were as follows:

1. NOSE/THROAT: number of days of nose and/or throat

soreness/pain in the sinus area and/or runny nose.

2. COUGH: number of days of hoarseness and/or cough.

3. CHEST: number of days of deep chest cough, wheezing and/or breathlessness.

4. EPISODES: number of discrete episodes of acute respiratory illness. A discrete episode is defined as one or more days of nose, cough or chest symptoms, preceded by and succeeded by at least one symptom-free day.

5. DAYS: the total number of days on which one or more of the symptoms nose, cough and chest were noted.

Respiratory symptoms were recorded and summarized for the pre-supplement, supplement and post-supplement periods (see fig.5. 1).

5.3.7 Diet Methodology

Three methods were initially considered to assess dietary intake of retinol and ß-carotene. They were: diet diary, diet history and food frequency questionnaires. Diet histories provide means of averaging nutrient intakes over a long period of time; however, the range of foods reported is subjective and prone to recall bias. In the food frequency method, the range of foods is specified, but because of the limited number of foods, an estimate of the total daily intake of a nutrient cannot be made in absolute terms. The range of foods recorded in diet diaries is more complete;

recall is not a problem and total intakes estimated. However, they represent only one point in time and are subject to bias from seasonal and other kinds of temporal variation. The relative merits of these methods for use in epidemiological studies have been discussed by Morgan et al (1978) and Abramson et al (1963). The results of the diet diary method were used in the analysis reported here.

5.3.8 Data Handling and Organization

Data were entered and organised on the Cyber computer at the University of Adelaide using the Scientific Information Retrieval (SIR) package. Analysis was performed using SPSS statistical procedures package. Data for each child included:

1. Blood analysis results: plasma retinol, a-tocopherol and ß-carotene.
   (a) initial, at the beginning of supplementation (T_0)
   (b) final, at the end of supplementation (T_1)
2. Dietary data: results of diet diary
3. General medical, family history, social and environmental information derived from the general questionnaire.
4. Height, weight and compliance data derived from dose cards.
   (a) initial (T_0)
   (b) final (T_1)
5. Respiratory information derived from diaries.

Data from Periods 3, 4 and 5 were summarised as pre-supplementary,

5-month and 11-month follow-up periods respectively. Dietary data were coded and analysed using the CSIRONET dietary analysis package with assistance from the Division of Human Nutrition, CSIRO, Kintore Avenue, Adelaide.

5.4 Results

Signed consent forms were received from parents of 153 children. Of these, six were excluded upon subsequent interview because of regular dietary supplementation with vitamin A during the preceding six months, or failing to meet the respiratory criteria. A total of 147 children were interviewed and entered into the trial.

5.4 .1 Number of Participants and their Attrition

5.4.1.1. Randomization and Comparability of Groups at Different Points in the Study

Table 5. 2 shows the composition of treatment and placebo groups with respect to the main potential covariates, after randomization, at five months (after completion of supplementation) and at 11 months (after completion of recording of respiratory symptoms). Treatment and placebo groups were similar after initial randomization when compared by mean age, sex, history of allergy and mean days of pretreatment respiratory symptoms, height for age and weight for height. A higher proportion of children with a history of lower respiratory illness (LRI) and frequent upper respiratory illness (URI) (appendix 5.5, Q20) were included in the placebo group (50% of placebo, 34% of supplemented group). However, this difference was not significant ($p = 0.13$).

The supplement/placebo group ratios for mean age, sex, history of LRI, URI and days of pre-treatment symptoms remained the same at both five and 11 months, indicating that no significant changes in the structure of the two groups had taken place over the trial period. A small increase (from 42 to 46%) was observed among those reporting history of allergy in the placebo population at 11 months. None of the differences observed between supplemented and placebo groups were significant at either five or 11 months.

Table 5.2

Comparability of Treatment and Placebo Groups

	N	Mean Age (Mths)	Sex (M) (%)	History of Allergy (%)	History of LRI (%)	History of URI (%)	Mean Presupplementary Days of Respiratory Symptoms
Initial							
Supplement	76	39.2	38(50)	30(39)	26(34)	25(33)	77.7
Placebo	71	39.4	35(49)	29(41)	36(50)	35(49)	71.3
	147 (100%)	39.3	73	59	62	60	74.5
5 Months							
Supplement	69	39.9	34(48)	27(39)	23(33)	22(33)	77.6
Placebo	65	37.5	32(50)	27(42)	32(50)	32(50)	72.5
	134 (91%)	38.7	66	54	55	54	75.1
11 Months							
Supplement	59	40.7	28(47)	22(37)	19(32)	18(31)	79.8
Placebo	48	36.0	23(48)	22(46)	24(50)	24(50)	76.6
	107 (66%)	38.4	51	44	43	42	78.2

5.4.1.2 Causes of Attrition

Dropouts were defined as those not completing 4/5 months of respiratory diaries at five-month follow-up, and those not completeing 10/11 months of diaries at 11-month follow-up. Table 5.3 shows the number of dropouts by cause.

Table 5.3
Number of Dropouts by Cause

No.	Cause
5	Discontinued
2	Moved away
6	Failed to return diaries at 5 months
27	Failed to return diaries at 11 months

The dropout rate of 8% during the first five months of the study (supplementation period) and 27% during the second six months was largely due to failure to return diaries. A vigorous effort was made through repeated telephone contacts with mothers to encourage their return. However, because of the demands of the vaccine trial, this period was the fourth 6 month period in which parents had been requested to keep diaries, and although they were highly motivated and a good rapport with staff was maintained throughout the trial, some fall-off in participation was inevitable.

5.4.2 Side Effects of Supplementation

Five children discontinued participation in the vitamin A study

because of illness believed by parents to be associated with the supplement. Symptoms reported were diarrhoea, vomiting and irritability. Of these children, four had been taking placebo. The fifth, although taking the vitamin A supplement, had a low plasma retinol value (1.03 umol/l) when starting supplementation. It was concluded that for these reasons, and the non-specific nature of the symptoms reported, the illnesses were due to other causes coincident with supplementation and not due to hypervitaminosis A.

In order to detect any side effects amongst those continuing in the study, parents were asked the question "Has this child been well since he/she began the medicine?" during their visit to the Ingle Farm Health Centre to collect their second bottle of supplement. Twelve parents reported a range of childhood illnesses. Of these, only one case of irritability was continuous over the period of time in which the vitamin A syrup was taken. Plasma retinol values for this child at the end of the supplementation period were well within the normal range (1.54umol/l). It was concluded that the problem was due to other causes.

5.4.3 Compliance and Validity of Variables

5.4.3.1 Respiratory Symptom Diaries

As discussed above, the number of completed respiratory diaries dropped from 91% to 68% at the conclusion of follow-up. It was of interest to determine whether the quality of recording also declined and, if so, if it was comparable in both groups. As part of the vaccine trial, nurses classified diaries as they were being coded according to quality of record keeping in

the following categories:

1. Record keeping excellent
2. Some problems with consistency and completeness
3. Major problems with consistency and completeness.

Table 5.4
Assessment of Respiratory Diary Quality

% of Diaries	5 Months		11 Months		Vaccine Trial*
	Placebo	Supplement	Placebo	Supplement	
N	65	69	48	57	1273
Excellent	97.4	100	97.4	100	89
Some problems	1.3	0	0	0	10
Major problems	1.3	0	0	0	2

* described in section 5.3.1

As shown in Table 5.4, the quality of recording in both supplement and placebo groups was very high and similar in both groups. In the placebo group, only two diaries with some problems were reported at 11 months and none in the treatment group, indicating little fall-off in quality over the trial period.

5.4.3.2 Vitamin A Supplementation Schedule

In order to ascertain what percentage of the dose children were actually taking, parents received a card for each child (appendix 5.6), on which they were asked to record a tick for each dose administered. Mothers were asked to bring dose cards and supplement bottles when visiting Health

Centres to receive their second supplement bottle and again at their concluding visit. The level in the bottle was checked against the number of ticks on the card, and any discrepancies queried. The percentage of the total dose taken was calculated from the card. Results are shown in Table 5.5.

Table 5.5

Compliance with Vitamin A Supplementation Schedule

% dose received	Number of children
0	3
50-74	8
75-99	6
100	119
	136

This method of assessing compliance relies on voluntary co-operation of parents. Although it is possible that dose cards may have been ticked indiscriminately and bottle contents discarded, the level of motivation in this voluntary population argues that the reporting was done honestly and a high level of compliance with the dosage schedule was maintained. Those taking 50% or more of the dose of vitamin A syrup (all of the vitamin A supplemented group) were included in the analysis as having received supplementation.

5.4.3.3 Diet Diaries

Of 134 diet diaries which were distributed to participants (diaries were distributed at 5 months), 122 (91%) were completed and returned. The quality of recording was good. Ninety-nine per cent of diaries contained

records over three days, complete descriptions of foods including brand names in many cases and estimates of quantities which were appropriate to the food and age of the consumer. The experience of filling out two previous diet history forms plus the prompts for easily forgotten foods and food descriptions provided at the two subsequent interviews may have contributed to the high quality of recording.

It should be noted that most diaries were filled out over a three-day period (Sunday, Monday, Tuesday), two weeks after Easter. Consequently the intake of sweets and chocolate was high. Thus the diet recorded may be biased towards low representation of so-called "healthy" foods. However, the recording of frequent intake of these and many other types of low nutrient value snack foods was a feature of this method, and argues for its presenting a truer picture of the diet than the history or frequency methods .

Mean values of the major nutrients estimated from the diet diaries are shown in Table 5.6. Comparison with mean daily intake values for energy, total protein, fat, carbohydrate, iron, vitamin C, vitamin B6, vitamin E and A from other studies shows close agreement. Comparison with the recommended daily allowances for these nutrients shows that the study population was consuming, on average, an adequate diet with respect to these nutrients.

Table 5.6

Mean daily intake of major nutrients estimated from 122 diet diaries in this study, compared with results of other surveys and RDA

Nutrient	This study (1-5 yrs)	RDA[1] (1-3 yrs)	HANES[2] (1-3 yrs)	OWEN et al[3] (2-3 yrs)	STUCKEY et al[4] (3-7 yrs)
M. Joule	5.6	5.5	6.2	5.1	7.7
Protein (g)	44.4	23	54.6	40	59
Total fat (g)	54.2	-	-	50	79
Total CHO (g)	173.2	-	-	180-200	230
Iron (mg)	6.6	15	7.7	6-7	10.6
Vit. C. (mg)	84.5	45	80.6	70	90
Vit. B6 (mg)	0.84	0.9	-	-	-
Vit. A (ugre) (total)	763	400	984	1020	764
Vit. E (mg)	2.96	5	-	-	-

[1] Recommended Daily Allowances, Food Nutrition Board, Ninth Edition, 1980, National Research Council, National Academy of Sciences, Washington DC.

[2] Health and Nutrition Examination Survey, 1971-74, United States Dept. of Health, Education and Welfare, Hyattsville, Maryland, 1977.

[3] Owen, G.M., Kram, K.M., Garry, P.J., Lowe, J.E. and Lubin, A.H. A study of nutritional status of preschool children in the United States, 1968-70.

[4] Stuckey, S.K., Darnton-Hill, I. Dietary intakes of five-year-old children in an outer Sydney suburb. Food and Nutr. Notes & Revs. 37, No. 3, 109-114, 1980.

Mean daily intakes of vitamin E and iron, however, were below the recommended daily allowances. Vitamin E in the diet is measured as

total tocopherols in this study. The intake of a-tocopherol, the most active form, is likely to be lower than this, to an extent depending on the dietary source of the vitamin - largely vegetable oils. Thus, corn oil contains total tocopherols at 100mg/100g, of which only 10% is a-tocopherol, while 90% of the total tocopherol content of safflower oil is a-tocopherol. Other tocopherols have only 10-50% of the activity of this form. Since different types of vegetable oils are not coded separately in the dietary analysis programme, it is not possible to reliably estimate the vitamin E intake from these data.

Mean daily intake of vitamin A in the unsupplemented group is well above the recommended daily allowance, although it is below intakes found in other studies. The relative contribution of the supplement and dietary ß-carotene to total vitamin A intake is considered in Section 5.4.5.2.

5.4.4 Blood Analyses

5.4.4.1 Mean Values of Plasma Retinol, ß-carotene and a-Tocopherol

Results of blood analyses are shown in Table 5.7. Blood samples were obtained from 95% (122) of children at the beginning of supplementation and 89% (93) at the end of supplementation. Plasma retinol values for all children in the study (range 25-97ug/dl) were in the normal to high range as defined by the United States Interdepartmental Committee on Nutrition for National Defence (ICNND) and above that level at which exhaustion of liver stores prior to a drop in plasma levels can be inferred (10-40ug/dl) (Flores et al, 1984).

The range (23-145ug/dl) and mean levels (66ug/dl) of plasma ß-carotene were low when compared with the "normal" range quoted by O'Brien (1969) for this age group (100-150ug/dl) and compared with mean values for 1-12-year-old children reported by Farrell (107ug/dl, 1978). However, plasma ß-carotene fluctuates diurnally depending on dietary intake. These samples were taken prior to the main meal of the day.

Table 5.7

Mean Plasma Vitamin Values in Supplemented and Unsupplemented Groups

	Retinol ug/100 ml.		ß-Carotene ug/100 ml.		a-Tocopherol (mg/100ml.)	
	To[1] (SE)[2]	Ti (SE)	To (SE)	Ti (SE)	To (SE)	Ta (SE)
Placebo	49.3 (1.7)	48.7 (1.4)	67.1 (3.2)	55.8 (3.2)	0.597 (0.019)	0.587 (0.025)
Supplement	48.41 (1.4)	48.4 (1.4)	63.9 (3.2	63.9 (2.7)	0.588 (0.018)	0.583 (0.030)
Overall	49.00 (1.1)	48.7 (1.4)	66.0 (2.1)	60.12 (2.1)	0.593 (0.013)	0.585 (0.017)
N	128	93	123	50	126	93

[1] To = presupplement
Ti = postsupplement

[2] SE = standard error of the mean.

Mean a-tocopherol (0.59mg/dl) was similar to that observed by Farrell (1978) in inner city children (0.55mg/dl) and suburban children (0.63mg/dl), although the minimum observed in this study was lower

(0.19mg/dl compared with 0.3mg/dl). Farrell reports a lower range of tocopherol values in children than in adults. Because of the high correlation between plasma tocopherol and total lipids, some authors have argued the necessity of expressing plasma tocopherols as a function of the latter. Farrell, however, found a much weaker correlation between the two in children (r=0.4, p= 0.05) than in adults (r=0.71, p= 0.001) possibly reflecting different dietary patterns in children.

5.4.4.2 Correlation Between Plasma Levels and Dietary Intake of Vitamins A and E

Plasma levels of vitamins were correlated with dietary sources only in the case of vitamin E (r=0.22, p=0.012, N=102). This is in agreement with other reports (discussed earlier) that plasma retinol is independent of dietary sources, except in clinical deficiency (<20ug/100ml).

5.4.5 Respiratory Symptoms

5.4.5.1 Correlation Between Periods

Nose/throat, cough and chest symptoms were measured consecutively over three 6-month periods encompassing two winters and a summer. Correlations between symptom variables measured over these three time periods are shown in Table 5.8. For the same symptom type, data from different periods were highly correlated. Pearson product moment coefficients ranged from 0.4 to 0.7. Seasonal differences did not change the strength of the relationship as summer-winter associations were as strong as winter-winter association.

Correlations between symptoms of different types were also significant, although weaker. Thus cough symptoms in period 3 were correlated with nose symptoms in period 4 (r=.24,p<001) and period 5 (r=.15, p=.047).

Table 5.8

Association Between Respiratory Symptoms in Period 3(1) and Those in Subsequent Time Periods (Pearson's Correlation Coefficients)

	Period 4(2)		Period 5(3)	
	r	N	r	N
Nose/throat	0.41	154	0.49	123
Cough	0.40	153	0.47	123
Chest	0.68	153	0.67	122

All correlations are significant at p<.001.

(1) 6-months prior to supplementation

(2) Supplementation period

(3) 6 months post-supplementation.

The possibility must be considered that the consistency of a child's experience from period to period represents a recording bias on the part of parents. However, the same relationship was observed in the more objective measures of number of GP visits and days of prescription drug ingestion and argues that it does represent a true consistency of morbidity from one period to the next. This finding has the following implications:

(1) a child experiencing frequent respiratory illness is likely to go on

experiencing it (it is not the result of a 'bad' winter);

(2) a population of children at risk or highly susceptible can be identified;

(3) up to 50% of the variance in these symptoms can be accounted for by factors which are continuous between periods. This has important aetiological implications, suggesting that factors such as genetic susceptibility, pre-existing underlying disorders and long-term nutritional and environmental factors play a highly significant role in determining morbidity, thus downgrading the relative importance of seasonal changes in exposure to infectious organisms.

5.4.5.2 Effects of Supplementary Vitamin A

In assessing the effects of vitaminA supplementation, three questions have been considered:

1. Was the dose successful in significantly changing dietary intake of vitamin A?
2. Was supplementation reflected in a change in plasma retinol values?
3. Did the supplemented group experience a drop in mean days or episodes of respiratory symptoms when compared with the placebo group?

5.4.5.2.1 Effect of Vitamin A Supplementation on Mean Daily Intake of Vitamin A

A comparison of mean daily intake of retinol, ß-carotene and total vitamin A in supplemented and unsupplemented groups is shown in Table

5.9. Total retinol intake for each case is equal to dietary retinol plus % compliance (proportion of vitamin A doses taken as indicated on the dose card) times daily dose of vitamin A (450ug retinol equivalents).

Table 5.9

Total Dietary Intake of Retinol and ß-carotene

	Mean daily intake of Vitamin A (ug retinol equivalents)		
	Retinol[1]	ß-carotene	Total Vitamin A
Placebo	394	369	763
Supplement	701	405	1106
% difference	44	8	31

[1] includes supplementary retinol.

Total daily intake of vitamin A in the placebo group was found to be derived equally from preformed retinol sources and ß-Carotene sources, a result which has been found for other Western populations (Goodhart and Shils, 1973). Vitamin A supplementation was effective in almost doubling the daily intake of retinol and increasing the total vitamin A intake (retinol + ß-carotene in retinol equivalents) by one-third.

5.4.5.2.2 Effect of Vitamin A Supplementation on Plasma Values

Table 5.7 shows mean plasma retinol values before and after supplementation. Plasma retinol was unchanged in the supplemented group at the conclusion of supplementation, although the placebo group

experienced a slight (non-significant) drop. This result indicates that plasma values were independent of diet and, by inference, suggesting that liver stores were adequate. The high mean plasma retinol of 48ug/dl also supports this.

5.4.5.2.3 Effect of Supplementary Vitamin A on Respiratory Symptoms

Mean days of respiratory symptoms over 11 months are shown for Vitamin A supplement and placebo groups in Table 5.10. Days of nose symptoms and chest symptoms were 12% and 9% lower (respectively) in the supplement group. The differences were not significant, however. Days of cough symptoms were 12% higher (again not significant). In the summary variables, episodes of all respiratory symptoms were 19% lower (significant at the 5% level) but there was no difference in days of all symptoms.

Table 5.10

Comparison of Respiratory Symptoms in Vitamin A Supplemented and Placebo Groups

	Nose (days)	Cough (days)	Chest (days)	Episodes of all Symptoms	Days of all symptoms
Placebo	62.5	28.3	15.1	8.0	72.7
N	51	51	51	52	54
Vitamin A	54.7	32.2	13.7	6.5	72.7
N	51	51	51	53	53
Overall	58.5	30.3	14.4	7.2	72.4
N	102	102	102	107	107
% change	-12	+12	-9	-19*	+.5

* = significant at 5% level (T test)

5.4.5.3 Effect of Stratification by Respiratory Morbidity History

The hypothesis central to the selection of children for the trial was that those with increased frequency and severity of respiratory infection may benefit more from vitamin A supplementation than those with lower levels of morbidity. Frequent infections, may exacerbate marginal vitamin A status, which in turn contributes to more frequent infections. It was therefore of interest to stratify children according to their prior experience of respiratory morbidity and to examine the effectiveness of vitamin A

supplementation within these sub-groups. The study population was stratified by pretrial morbidity (recorded as part of the vaccine trial) and by history of lower respiratory illness and asthma, variables derived from the general questionnaire.

Stratification by days of symptoms during the 6 months prior to the study showed no modification by pre-trial morbidity of the effectiveness of supplementation. However the study population was selected on the basis days of more than 15 days of cough symptoms (or three episodes of respiratory illness) during the three month period prior to the study. Pretrial morbidity measured in this way would thus be uniformly high and may not provide enough variability to show an effect.

The population was further stratified on the basis of prior experience of bronchitis, pneumonia, persistent cough, croup, whooping cough (termed lower respiratory illness in this thesis) and of asthma, hayfever, eczma or food allergy (termed allergy) . A difference in response to vitamin A between children with and without a history of illness then emerged (Table 5.11) .

Children with a history of allergy and lower respiratory illness showed greater reduction in symptoms associated with vitamin A supplementation than children without such a history. Children with a history of allergy had reductions of 16 - 29% in episodes, days, nose, throat and cough (although none was significant the power of the study to detect significant differences in these variables was very low). Children with a history of LRI had a 25% reduction in number of episodes significant at the 5% level. No reduction was observed in children without such a history. Children with and without LRI

history had similar mean days of symptom morbidity (72.6 SEM 6.7 and 72.8 SEM 8.0 respectively).

Table 5.11
Percentage Difference between Supplemented and Placebo Group Means, Stratified by History of Lower Respiratory Illness & Allergy

	History of Allergy(1)		History of LRI(2)		Total Population
	No	Yes	No	Yes	
N	63	4	39	68	107
Episodes	-11	-25	0	-25*	-19
Days	17	-16	4	-1	0
Nose/Throat	4	-29	-10	-14	-12
Cough	57	-27	-2	25	+12
Chest	-25	1	81	-29	-9

(1) Asthma, hayfever, eczema or food allergy

(2) Bronchitis, pneumonia, croup, whooping cough, persistent cough.

Source: parent questionnaire.

*p <.05

Stratification, by reducing the effective sample size, reduces the power of the study to detect a significant difference. It also increases the possibility of differences between treatment groups in infrequent variables. However, comparison of the major potential confounding factors of age, days of pre-supplementary morbidity, allergy and frequent upper respiratory illness showed no differences between treatment and placebo groups in LRI and no LRI children.

Thus there appears to be a consistent benefit derived from vitamin A supplementation for children with a history of lower respiratory illness expressed as a reduction in incidence of all respiratory symptoms.

5.4.5.4 Correlation Between Plasma Levels of Vitamins A and E and Respiratory Outcome Variables

Table 5.12 shows the correlation between plasma retinol, ß-carotene and a-tocopherol and episodes and days of respiratory symptoms. No significant correlations were found between retinol, ß-carotene and respiratory symptoms, either at the beginning or conclusion of the supplementary period, suggesting that plasma vitamin A is not affected by increasing frequency of respiratory symptoms, nor does it contribute to it.

Episodes of respiratory symptoms were weakly, but significantly correlated with plasma vitamin E levels at the conclusion of the supplementary period however, suggesting that increased frequency of infections may have reduced plasma a-tocopherol. This was confirmed when change in plasma a-tocopherol was plotted against episodes of respiratory infection. The association had a Pearson's Correlation coefficent of -0.27 ($p=0.009$, $N=72$). Neither of these associations were found with days of respiratory illness. Possible mechanisms for this effect include increased utilization due to the turnover of membrane lipoprotein with replacement of virus-destroyed respiratory mucosal cells or increased utilization due to the anti-oxidant role of vitamin E. The absence of an association with plasma retinol or ß-carotene suggests that overall dietary intake of vitamin A is not associated with respiratory morbidity.

Table 5.12

Pearson's Correlation Coefficients for the Association between Plasma Vitamins and Episodes and Days of Respiratory Symptoms at the Beginning and End of Supplementation.

Plasma		Initial			Final	
Vitamins	(N)	Episodes	Days	(N)	Episodes	Days
Retinol	103	0.13	0.04	76	-0.07	-0.02
ß-carotene	98	0.04	0.12	69	-0.02	-0.01
a-tocopherol	100	0.11	0.06	76	-0.22*	-0.11

* p=0.033

5.4.6 Social and Environmental Factors: Multivariate Regression Analysis

A study of other potential co-variates of respiratory symptoms was made with the purpose of detecting factors which could confound a nutritional effect, and of estimating the magnitude of the correlation of respiratory variables with vitamin A relative to other known correlates.

Information on a number of socio-economic, environmental, medical and family history variables which have been shown in other studies to be related to respiratory illness was derived from the questionnaire (Appendix 5.5). These variables were in the categories of respiratory history of the child and family, environmental and socio-economic factors, factors relating to exposure (contact with potential

sources of virus transmission), family composition and the behaviour of parents in seeking treatment for respiratory symptoms in their child. The variables and their type were as follows:

		Type
A.	Medical:	
1.	History of Frequent Upper Respiratory Illness: persistent runny nose, sinus trouble, tonsillitis, frequent ear infections.	Dichotomous
2.	History of Lower Respiratory Illness : bronchitis, pneumonia, croup, whooping cough, persistent cough.	Dichotomous
3.	History of allergy : asthma, hayfever, eczema or food allergy.	Dichotomous
4.	History of measles	Dichotomous
5.	Age	Continuous
B.	Family History:	
1.	History of adult lower respiratory illness : history of persistent cough, wheezing, bronchitis, pneumonia, emphysaema or other chest illness prior to last 12 months amongst adults.	Dichotomous
2.	History of sibling lower respiratory illness : history of persistent cough, wheezing, bronchitis, pneumonia, croup or other chest illness amongst siblings.	Dichotomous

C. Environmental:

1.	Type of heating/cooking (gas or not)	Dichotomous
3.	No. of smoking parents during 1st 2 years of life.	Continuous
4.	No. of parents currently smoking	Continuous
5.	Airconditioning :presence or absence	Dichotomous

D. Socio-economic:

1.	Mother's education: years full-time	Continuous
2.	Father's education: years full-time	Continuous
3.	Mother's occupation : Congalton 7-point scale	Ordinal
4.	Father's occupation : Congalton 7-point scale	Ordinal

E. Exposure:

1. Episodes of adult upper respiratory illness : Continuous
   episodes of sore throat, runny nose or cold-like symptoms in past 12 months amongst adults at home.
2. Presence of adult lower respiratory illness : Dichotomous
   persistent cough, wheezing, bronchitis, pneumonia, emphysaema or other chest illness during the last 12 months amongst adults. Dichotomous
3. Episodes of sibling upper respiratory illness : Continuous
   episodes of sore throat, runny nose or cold-like symptoms in past 12 months amongst siblings this year.
4. Presence of sibling lower respiratory illness : Dichotomous
   persistent cough, wheezing, bronchitis, pneumonia, croup or other chest illness during the last 12 months amongst siblings.

F. Help Seeking Behaviour - Tendency to seek treatment for: Score

1.	Continuous runny nose	0, 1, 2
2.	Sore throat	0, 1, 2
3.	Frequent cough	0, 1, 2
4.	Clears throat	0, 1, 2
5.	Irritated and tired	0, 1, 2
6.	Repeats words.	0, 1, 2

where a score of 0 = regards as normal part of growing up

1 = applies home remedy or something from chemist

2 = consults doctor

G. Family Composition:

1.	Number of siblings 0-2 years	Continuous
2.	Number of siblings 3-4 years	Continuous
3.	Number of siblings 5-10 years	Continuous
4.	Number of siblings 11-18 years	Continuous

These variables were entered into a multiple linear regression model with backward elimination: after entry of all variables, those with the largest probability of F were then removed sequentially, if the latter probability was greater than 0.05. Residuals were tested using a normal probability distribution. The variables entered into the model, and their coefficients are shown in table 5.13 and 5.14.

Table 5.13
Medical and Family History, Social and Environmental Variables selected in a Backwards Multiple Linear Regression Model with Episodes of Respiratory Symptoms as the Dependent Variable.

Variable	Regression Coefficient	T-Statistic	Significance of T	Partial Correlation Coeff.
VitA Supplement	-2.07	-2.73	0.008	-0.31
History of Adult LRI	2.51	2.90	0.005	0.33
Presence of Adult LRI	-2.55	-3.00	0.004	-0.34
Episodes of Adult URI	0.43	2.77	0.007	0.32
Episodes of Sib URI	-0.39	-2.01	0.048	-0.24
Measles	2.85	2.03	0.046	0.24
Airconditioning	-3.37	-4.16	0.000	-0.45
Treat Nose Symptoms	1.73	2.30	0.024	0.27

The model explained 37% of the variance in respiratory episodes (R-square=0.44, adjusted R-square=0.37). A similar model, using stepwise entry of variables resulted in fewer variables being included, but explained less of the variance (adjusted R-square= 0.25). The results of backwards multiple regression using days of respiratory symptoms as the outcome variable is shown in table 5.14.

The model explained 35% of the variance in symptom days (R-square=0.44, adjusted R-square=0.35). Stepwise linear regression resulted in a similar, but less explanatory model (adjusted R-square= 0.18).

Table 5.14
Medical and Family History, Social and Environmental Variables selected in a Backwards Multiple Linear Regression Model with Days of Respiratory Symptoms as the Dependent Variable.

Variable	Regression Coefficient	T-Statistic	Significance of T	Partial Correlation Coeff.
Mother's Education	-9.9	-3.0	0.004	-0.35
Father's Occupation	-9.4	-2.2	0.034	-0.26
Mother's Occupation	-13.0	-3.3	0.001	-0.38
No of Preschool Sibs	21.8	1.8	0.073	0.22
No of Adults at Home	82.3	2.3	0.021	0.28
History of Adult LRI	40.3	3.3	0 001	0.38
Presence of Sib LRI	-28.6	-2.2	0.032	-0.26
Gas Heating or Cooking	-29.6	-2.3	0.026	-0.27
Treat Irritability	-14.3	-1.9	0.056	-0.23
Treat Throat Symptoms	35.1	1.73	0.088	0.21
Treat cough Symptoms	30.9	2.0	0.048	0.24

5.4.6.1 Vitamin A Supplementation

The treatment variable was entered into the multivariate analysis, in addition to the variables mentioned above, for two purposes: to check that the difference between the two groups in the confounding factors history of LRI and URI (section 5.4.1.1) was not contributing to the treatment effect observed, and secondly to determine the magnitude of the contribution of the treatment variable to overall variability.

Vitamin A supplementation was a significant predictor of respiratory episodes after all major potential confounding factors had been included in the regression. Of those differences which remained between groups after randomization, none were responsible for the difference in outcome observed between treatment and control groups (there remains the remote possibility of unmeasured confounding differences between the two groups, as in all randomized controlled trials). The contribution of the supplement variable to the overall variance, however is not great. In the stepwise regression model, vitamin A supplementation contributed 5% of the overall variance. Vitamin A supplementation therefore, while it may have significantly influenced respiratory episodes, was just one of many factors also correlated with this outcome. As observed earlier, symptom days of respiratory illness were not associated with the treatment variable, again, indicating that the effect of vitamin A supplementation on respiratory morbidity in this population is minor. In conclusion, multiple regression including the major known social and environmental confounders for URI confirmed a significant effect of medication on episodes but not days of respiratory illness. The magnitude of the effect of supplementation is only modest compared with contributions from other variables.

5.4.6.2 Socio-economic, Behavioural Factors and Family History as Predictors of Respiratory Symptoms.

Only one third of the risk factors listed in the categories described above, proved to be significant contributors to variability of respiratory symptoms in this population. Variables in the category of socio-economic status were the most consistent predictors. Mother's Occupation and Education and Father's Occupation were contributors to days of symptoms (partial

correlation coefficients, r= -0.38, -0.35 and -0.26 respectively). The direction of the relationship was consistently negative, and this is accordance with a large body of literature which reports a strong social class gradient for respiratory illness (Lee and Holland, 1978; Monto and Ross, 1977). The apparent protective effect of air conditioning on episodes of symptoms is difficult to explain unless, in this relatively homogeneous population, presence or absence of air conditioning is a proxy for socio-economic status.

Of the exposure variables, Presence of Adult LRI and Episodes of Adult URI and Sibling URI in the past 12 months were contributors to respiratory episodes, however they were not consistent in the direction of their effect and no exposure variables were contributors to days of respiratory symptoms. History of Adult Lower Respiratory Illness was a consistent contributor to both episodes and days (r=0.33, r=0.38, respectively). Sibling history of LRI did not reach significance as a predictor in this model. Parent's history of LRI (chronic bronchitis, asthma) has been reported as a predictor of chronic bronchitis in children by Higgins and Keller (1975). It is interesting that neither the current smoking habits of parents, or those during the first two years of life, were predictors of episodes and days of respiratory illness in this population.

The 'tendency- to- seek- treatment' variables (see general questionnaire, appendix 6), reflect a parent's tendency to see minor symptoms as important and worthy of seeking external help. They therefore reflect a parent's awareness of the child's state and perception of the importance of respiratory symptoms relating to his or her health. This perception is relevant to the study, because the outcome used in the trial was a symptom variable reported by parents. The tendency- to- seek- treatment variables

should be a measure of the extent to which the outcome variable was influenced by differences in parents perception of the importance of respiratory symptoms. The tendency- to- treat- nose symptoms was a predictor of episodes (r=0.27) and the tendency-to-treat-throat and cough symptoms were predictors of symptom days (r=0.21, 0.24 respectively), suggesting that some variation in these variables was due to differences in their perceived importance amongst parents. The negative contribution of tendency to treat irritability to symptom-days, may reflect an inclination of parents to ignore behavioural symptoms as noteworthy indicators of ill-health.

5.5 Discussion

The objective of the study was to determine whether changing vitamin A status by oral supplementation would affect respiratory morbidity amongst children who experienced frequent respiratory symptoms (i.e.were 'respiratory prone'). The underlying hypothesis assumes three conditions:

1. that these children would be marginally vitamin A deficient (inadequate vitamin A reserves to provide a minimal protective period - WHO, 1976)
2. that their vitamin A status would benefit from increased dietary intake
3. that the benefit would be expressed in a reduction in episodes and days of respiratory symptoms.

On the basis of both dietary intake and plasma retinol levels, it is unlikely that the population is marginally vitamin A deficient. Thus mean daily

intake of vitamin A in the placebo group was 763 ug retinol equivalents(re), almost twice the recommended daily allowance for this age group. This margin seems adequate to allow for an increased requirement due to frequent infections, unless a major problem in absorption was encountered.

A mean plasma retinol of 49ug re/dl also is above levels associated with marginal liver reserves (<30ug/dl: Flores et al, 1984). No change was observed in the supplemented group at the conclusion of supplemenation, despite an increase in total vitamin A intake of 45%. No change would be expected if dietary intake was already high and liver reserves were adequate.

Given that the two prior conditions of the underlying hypothesis were not met, it was surprising that a third - namely a reduction in respiratory morbidity in response to supplementation- appeared to occur. Thus a 19% reduction (significant at the 5% level) was observed with vitamin A supplementation. The effect was observed in episodes of respiratory symptoms, however not observed in symptom days. This apparent inconsistency may be a result of the limited size of the study population. Retrospective power calculations showed that in a study population of this size, there was a 91% chance of detecting a 25% difference in episodes (a=0.05), but only a 64% chance of detecting a similar difference in symptom days. A larger population size was therefore necessary in order to be able to detect an effect in symptom days. In a multiple linear regression model, vitamin A supplementation remained as a predictor of episodes of symptoms when major medical and family history, socio-economic and environmental risk factors were included, although its contribution to the overall variance was small (5%).

Children with a history of allergy and lower respiratory illness appeared to benefit more than those with no such history (again, possibly due to problems of power, significant reductions occurred in episodes and not days of respiratory symptoms) The variable 'lower respiratory illness' as used in this study is very diverse- including bronchitis, pneumonia, croup, whooping cough and persistent cough (croup and whooping cough are not normally considered as affecting the lower respiratory tract) It is a parent report of a doctor's assessment and therefore subject to problems of recall bias and differential diagnosis. Nevertheless, it does point to a group who may have a greater requirement for vitamin A.

A number of studies have shown long term sequelae (increased incidence of respiratory symptoms and deficits in lung function) in groups of children who suffered LRI in infancy (reviewed by Samet,1984). Severe viral respiratory infection is known to cause extensive damage to the muco-ciliary lining of the respiratory tract. If such damage is responsible for enhanced susceptibility to respiratory infection subsequently, then increasing vitamin A availability at the tissue level may interrupt this cycle. A reduction in plasma levels of vitamin E after frequent respiratory illness as observed in this study may further increase vitamin A requirements in these children.

It must be emphasized that this study was not designed to test differences in response to vitamin A supplementation between children with and without a history of lower respiratory illness. Although, as far as could be determined, major confounding factors were equally represented in both groups with and without history of LRI, other factors such as unknown

confounders, variability in parental recall, disease definition and small sample size may have influenced the observed results. These findings were regarded as preliminary, but worthy of investigation. As a result of this study, the focus of investigation changed from that of the respiratory prone child, as defined by frequency of recent infection, to respiratory proneness as defined by early history of LRI. A study was planned in which this group of children was more tightly defined, and their vitamin A status examined.

5.6 Summary and Conclusions

5.6.1 A randomized, controlled trial of vitamin A supplementation was carried out on 147 preschool children who had experienced more than 3 episodes of respiratory symptoms and 15 days of cough during the preceding 3 months. The hypothesis tested was that children in this age group who have experienced multiple episodes of respiratory illness may be in a state of marginal vitamin A deficiency and that this contributes to continuing susceptibility to ARI.

5.6.2 A dose of 450 ug retinol equivalents (re)/day of retinyl palmitate was provided over a period of 5 months and respiratory symptoms recorded on a daily basis by parents for a further 6 months. HPLC analysis of plasma retinol, ß-carotene and a-tocopherol was performed on fingerprick blood samples taken at the beginning and end of supplementation. Dietary vitamin A determination was by parent-completed 3-day food record.

5.6.3 Mean daily intake of retinoids was 394 ug re/day in the placebo group and 701 ug re/day in the supplemenated group after adjustment for compliance. Total vitamin A intake in the placebo group was 763 ug re/day,

indicating that even without supplemenation, vitamin A intake was well above the levels generally considered necessary to maintain health (450 ug re/day).

5.6.4 At the conclusion of supplementation, plasma retinol was 48.4 and 48.7 ug/dl in the supplemenated and placebo group respectively. Plasma retinol did not change with supplementation and was not correlated with total dietary intake of vitamin A.

5.6.5 Respiratory symptoms were highly correlated from one 6 month period to the next, confirming the notion of "respiratory proneness" reported in other studies. This finding suggests that respiratory episodes are not independent events, but are a result of one or more underlying factors which are continuous from period to period.

5.6.6 Vitamin A supplementation was associated with a significant, 19% reduction in the total number of episodes of respiratory symptoms ($p<0.05$). Reductions in days of nose and chest symtoms of 12% and 9%, respectively were non-significant. A 12 % increase in days of cough in the supplemented group was also non-significant.

5.6.7 Stratification of symptoms in the placebo group by 6-month pre-trial morbidity did not show an enhanced effect of supplementation on respiratory symptoms in sicker children. Stratification by history of lower respiratory illness and allergy did show a greater benefit to such children. A 25% reduction in the number of episodes of respiratory illness (significant at the 5% level)was observed in 63 children with a parent reported history of prior lower respiratory illness (bronchitis, pneumonia, croup, whooping

cough or persistent cough).

5.6.8 Multiple linear regresssion was performed examining the relationship of medical history, family medical history, passive smoking, socio-economic status, family composition and other factors to acute respiratory morbidity. Of these, parental history of lower respiratory illness, family composition and some environmental factors were most strongly correlated with symptoms. The treatment variable remained a significant predictor of respiratory episodes after entry of all other variables into the model.

Conclusions related to vitamin A status and respiratory morbidity are as follows:

5.6.9 Evidence from dietary estimates and response to supplementary vitamin A indicates that these children are not in a state of marginal vitamin A deficiency.

5.6.10 Supplementation was nevertheless associated with a reduced incidence of respiratory symptoms, particularly in children with a history of lower respiratory illness (as defined in this study).

Chapter 6

Vitamin A Status and Respiratory Morbidity in Children with a History of Lower Respiratory Illness in Infancy

6.1 Introduction

The previous study established that respiratory- prone children in the community - defined by the frequency of recent respiratory symptoms - are not marginally vitamin A deficient. Nevertheless a subgroup of that population, loosely defined as those with a parent-reported history of pneumonia, bronchitis, whooping cough, croup and persistent cough - appeared to benefit from supplementation. Similar, but not significant results were found for those with a history of asthma, hay fever, eczema and food allergy. That children with a history of early lower respiratory tract infection constitute a special group who are particularly prone to respiratory illness has been shown in a number of studies. Samet (1984) in a review of the origins of chronic air flow obstruction in adults summarized reports which showed that children who experienced bronchitis, bronchiolitis and pneumonia in infancy have evidence of deficits in small airways function, reduced airflow, airtrapping and increased bronchial reactivity (the latter independent of history of atopy), 1-10 years later.

Subsequent studies have confirmed increased bronchial reactivity and diminished ventilatory function in these children (Breese Hall et al, 1984, McConnochie and Roghman, 1984, Mok and Simpson, 1984a,1984b) Mok and Simpson report more cough, tendency for colds to 'go to the chest', days off school for respiratory illness in the past year and general practitioner consultations in this group. The similarity of these effects in groups of children with bronchitis, bronchiolitis or pneumonia as the index diagnostic classification lead them to suggest that the category of LRI is not important in determining its long-term outcome (Mok and Simpson. 1984a). Possible

mechanisms for these effects are an underlying susceptibility to respiratory illness, present at birth, or injury due to viral infection during a period of rapid lung development, which consequently has long lasting effects.

Vitamin A has been shown to play a role in lung development. Both cellular retinol binding protein and cellular retinoic acid binding protein are present in foetal rat and human lung (Chytil, 1985) and levels peak during periods of maximum differentiation in rat lung (Ong and Chytil, 1976). Serum vitamin A levels are lower in preterm than fullterm infants (Shah and Rajalakshmi, 1984; Shenai et al, 1981; Brandt et al, 1978; Howells et al, 1984). They have been shown to be lower at birth in preterm infants who develop brochopulmonary displasia than those who do not, and to remain low for an extended period postnatally (Shenai et al, 1985). The authors speculate that the necrotising bronchiolitis and squamous metaplasia of vitamin A deficiency may influence repair of lung injury due to mechanical ventilation. Sub-optimal availability of vitamin A may enhance epithelial necrosis as a result of viral attack, and thereby contribute to its long term consequences.

Many of the studies of the long term effects of lower respiratory tract infections cited above used Respiratory Syncytial Virus infection or bronchiolitis as the index illness (Pullan and Hay, 1982; McConnochie, 1984; Mok and Simpson, 1984; Henry et al, 1983; Kattan et al, 1977; Sims et al, 1978). The long term sequelae of this illness and hence the respiratory prone state of children who experience it is thus well documented. Moreover, children hospitalised with bronchiolitis can be easily defined and identified using laboratory and hospital records. It was therefore proposed

to confirm the findings of the previous study in a population of children chosen on the basis of hospitalization for Respiratory Syncytial Virus infection in infancy.

A double blind, randomised, controlled trial of vitamin A supplementation was deemed again to be the study design of choice, because of the need to distinguish cause and effect in the relationship between vitamin A status and respiratory illness. The hypothesis tested was that changing vitamin A status (through oral supplementation) in these high risk children would reduce episodes of respiratory morbidity. Large numbers of 2-6 year old children had been hospitalized in infancy at the Adelaide Children's Hospital with RSV positive bronchiolitis and these could be selected from records provided by the Institute of Medical and Veterinary Science (I.M.V.S.).

6.2 Sample Size Considerations

Desirable sample size estimates were based on parameters from the previous study and are given in table 6.1. It was proposed to select a total population size of 250, thus giving a 95% chance of detecting a 25% difference in episodes, and 80% chance of detecting a 25% difference in total days of respiratory symptoms. Approximately 120 RSV positive cases of hospitalized LRTI were identified by IMVS each year over the required time period (1978-1982).

Table 6.1
Sample Size Required to Detect 25% Difference (a=0.05) in Mean Episodes and Days of Respiratory Symptoms

Variable	Difference	Standard Deviation	Sample Size (each group)	Power (%)
Episodes	1.8	3.8	80	85
			125	95
Total days	18.1	53.7	158	85
			125	75
Nose/throat	14.6	46.2	180	85
(days)			125	70
Cough	7.6	32.6	330	85
(days)			125	45

6.3 Method

6.3.1 Selection of the population

Records were obtained from the I.M.V.S. of nasopharyngeal swabs from children admitted to the Adelaide Children's Hospital during the years 1978 to 1982. All those which were positive for Respiratory Syncytial Virus by either culture or RSV-antigen immunofluorescence were selected. Hospital casenotes were searched to confirm the identity of the child, extract addresses and identify conditions which would lead to exclusion on medical grounds (section 6.3.2.1). Recruitment letters were sent out (appendix 6.1). Replies were followed with an initial telephone call in which the aims of the study were explained. An information sheet, general questionnaire and

informed consent from (appendix 6.3 - 6.5) were sent out and then a second telephone call made to arrange the first appointment. Home visits were made to respondents without telephones. At the second contact, a questionnaire was administered to determine any recent reasons for exclusion (appendix 6.6). Letters were sent to general practitioners of children who were entered into the trial (appendix 6.7).

6.3.2.1 Exclusions

Conditions which were considered reasons for exclusion from the trial were as follows:

Conditions likely to cause chronic respiratory symptoms, eg, cystic fibrosis

Cardio-pulmonary abnormalities

Major brain dysfunction eg, epilepsy, Down's syndrome

Vitamin A supplementation at >RDA more than 3 times weekly over past three months

Liver eaten more frequently than once per month

6.3.2 Study Plan

It was proposed to supplement children and follow up respiratory symptoms using a parent completed diary for a period of a year. At clinical examinations at the beginning and end of this period, children were weighed, measured, finger prick blood samples were taken, buccal

washings collected and lung function measurements made. A skin test, measuring response to challenge with a standard range of allergens was administered at the beginning of the study. Every three months subsequently, respiratory diaries were collected and supplement bottles reissued. This occurred at the ACH in a brief interivew, at which time parents were asked whether the child had been well. Home visits were arranged for those without transport. Telephone contacts were made in between visits to the hospital, so that contact with parents of participants was made every 6-8 weeks. The study plan is summarized as follows:

Nov, '85	Feb, '85	May, '85	Aug, '85	Nov, '85
Initial clinical exam	2nd bottle distribution	3rd bottle distribution Diet Diary distribution	4th bottle distribution	Final clinical exam

6.3.3 The Supplement

The supplement was formulated by Faulding & Co. Ltd., to deliver an average daily intake of 600ug retinol equivalents (re) of retinyl palmitate as a weekly dose of 4.2mg. A weekly rather than daily dose was chosen to improve compliance and to simplify distribution of supplement bottles. The dose was too low to be associated with side effects (section 5.3.3.3), however it is possible that proportionally more vitamin A may be absorbed or utilized when delivered as a daily rather than weekly dose. This

advantage was balanced against the problems of maintaining daily supplementation over a one year period in a population of young children who frequently reject medication of any kind. The practicality of the distribution of large numbers of bottles was also a factor in the decision. The formulation is given in appendix 6.9.

6.3.4 Randomization

Vitamin A supplement was randomly allocated 4 of 8 batch numbers and placebo was allocated the remaining 4 numbers. Supplement bottles were labelled with the appropriate batch numbers and the batch number code was retained by the manufacturer. The bottles were distributed sequentially according to batch number as children presented for the initial clinical examination.

6.3.5 Measurement of Respiratory Symptoms

Respiratory symptoms were measured using a parent completed diary as before, however the list of symptoms was expanded (appendix 6.10). Symptom variables derived from respiratory diaries comprised total days and episodes of the following symptoms:

Nose	sneezing
	runny/blocked
Throat:	hoarse

sore/scratchy

Cough: dry

chesty/moist

Breathing: wheezy

short of breath

rapid

Systemic: fever

vomiting

diarrhoea

muscle ache

Illness variables measured were:

At home sick

Dr. or outpatient visits

Hospital admissions

A similar list had been used in a trial of interferon prophylaxis of respiratory virus infection among adults (Douglas et al, 1985) where efficacy of treatment was assessed using daily recording of symptoms. Based on the constellation of symptoms present and their duration, shown diagrammatically in appendix 6.11, episodes had been designated as 'probable', 'uncertain' and 'doubtful'. Validation of this classification as an index of respiratory infection had been provided by viral culture of blood samples and serologically determined antibody response to a range of viral respiratory pathogens. On this basis, 29% of the probable episodes, 18% of the uncertain and 10% of the doubtful had shown evidence of viral infection by the pathogens tested.

In the present study, in addition to classifying episodes as 'probable',

'doubtful' and 'uncertain', days and episodes of symptoms were coded individually (appendix 6.11, 6.12, 6.14). This was felt to be important in case reduction of symptoms occurred at only one anatomic site (eg, nose or throat). In comparing the outcome in treatment and placebo groups, Mann-Whitney and median tests were used for all variables except total episodes and probable infections, for which a t-test was used.

6.3.6 Compliance

6.3.6.1 Respiratory Diaries

Parents were interviewed on receipt of each diary. This was checked for completeness (every day checked), consistency (eg, antibiotic taken but no doctor visit entered and evidence of casual entry (eg, every entry alike) and the parent questioned about any problems. Each month was then graded as follows:

1. good
2. some problems
3. not reliable/inadequate

6.3.6.2 Supplementation

Parents were issued with dosecards with the date of each weekly dose entered (appendix 6.15). This was ticked by the parent when the dose was actually taken. At interview, the number of ticks was compared with the level in the bottle and inconsistencies queried. It was emphasized that any lost or spilt medication should be recorded.

6.3.7 Dietary Assessment

Dietary intake was determined by 24 hour recall, diet frequency and 3-day diet diary methods. The 24 hour recall and frequency questionnaire were administered at initial and final interviews by the author. Food models used were 170 ml glass, 240 ml cup, a 9 cm square representing a cheese slice, a block of cheese with 0.5 and 1.0 cm slices indicated and a rice bowl (240 ml capacity). These models had been found to be most useful in the previous study. The 3-day diet diary was identical in format to that in the previous study and was administered only once, mid-way through the year. These methods were chosen in order to have both absolute and relative measures of vitamin A intake and to detect any change in intake, particularly in the placebo group over the period of the trial. Because of the high day to day variability in vitamin A intake (one serve of liver provides the RDA for 10 days), an averaging method was selected for the baseline and final measurement. Estimates of total intake are derived from the three day diet diary.

6.3.8 Biochemical Analyses

Analyses of plasma retinol were carried out as in the previous study. It was decided not to measure plasma ß-carotene because of the additional volume of blood required and the difficulty of obtaining it using the finger-prick collection method.

6.3.9 Pulmonary Function and Skin Tests

Lung function measurements were made at the beginning and end of the study. Forced vital capacity (FVC), Forced expiratory flow in 1 second (FEV1), Flow at 25% and 50% vital capacity (V25, V50), Forced expiratory flow at 25-75% vital capacity (FEF25-75) and Flow at functional residual capacity (VFRC) were obtained from forced expiratory manoeuvres before and after administration of bronchodilator. All children were subjected to skin tests as a measure of atopy. Response to ventolin inhalation (75-125mg/100ml) and to histamine challenge (0.03-10mg/ml) were taken as measures of bronchial hyperreactivity. A change of 10% predicted in FEV1 after bronchodilator inhalation and 20% of baseline FEV1 after histamine inhalation were taken as positive indications of hyperreactivity.

Children were tested by skin abrasion for responses to the following antigens: Ryegrass, Cocksfoot grass, Yorkshire fog grass, cat fur, dog hair, house dust and house dust mite (CSL Allergen Extract, D strength). A wheal of 1mm or more to one or more antigens was considered a positive response.

6.4 Results

6.4.1 Selection and exclusions

All IMVS records of nasopharyngeal swabs taken from patients who

were admitted to the ACH with bronchiolitis between 1978 (the first year that records were kept) and 1982 were examined and those who were either culture positive or had positive immunofluorescence tests were selected. Children selected in this way were excluded if they did not live in the metropolitan area, had underlying medical conditions (section 6.3.1) or were ineligible because of vitamin A supplementation. A list of medical conditions causing exclusion is given in appendix 6.15.

Table 6.2
Selections and Exclusions

No of records selected as above		643
not eligible:	out of town	98
	medical exclusions	33
	deceased	4
Initial recruitment letters sent		508
not eligible:	vitA intake criteria	15
losses:	untraceable	157
	refusals	128
	unable to attend	2
Total number of acceptances eligible for randomization		206

6.4.2 Compliance

The study protocol demanded compliance by parents in two activities: daily recording of symptoms and weekly supplementation of the child.

Compliance was assessed using criteria of diary quality and proportion of total dose taken.

6.4.2.1 Diary Quality

Each month of recording was assessed on a three point scale as follows:

1. good
2. some problems
3. unreliable

A list of problems which caused a rating of 2 is included in appendix 6.17. The quality score was summed over three months to give an average quarterly index of quality. Thus a perfect score for diary quality per quarter would be three. If only one month out of three had some problems, then the quarterly score was 4 and deemed acceptable. Scores higher than 4 in any one quarter were deemed unacceptable. The number of diaries of acceptable quality is shown in Table 6.3.

Table 6.3

Quality of Respiratory Diary Recording

Quarter	Q1	Q2	Q3	Q4
Acceptable	179	171	165	173
Unacceptable	19	26	21	13
Missing	8	9	20	20
	206	206	206	206

6.4.2.2 Dosage of Vitamin A

The number of doses actually consumed during each three month period was estimated from the dose record card kept by the parent and the amount of supplement remaining in the bottle at the conclusion of the period. A minimum of greater than or equal to 10/12 doses in the first and second quarter, 9/12 doses in the third quarter and 8/12 in the 4th quarter was deemed acceptable. The number of subjects whose compliance with the dosage schedule was deemed acceptable is show in table 6.4.

Table 6.4
Compliance with Supplementation Protocol

Quarter	1	2	3	4
Acceptable	179	173	167	142
Unacceptable	9	10	6	27
Missing	18	23	34	37
	206	206	206	206

6.4.2.3 Criteria for inclusion in study

Subjects whose mean diary quality or dosage of vitamin A were unacceptable according to the above criteria were deemed to have dropped out at the beginning of that quarter. Subjects who had taken 36/48 doses in total, and whose diary quality, averaged over 4 quarters was between 3.0 and 4.0 were considered to have completed the study. Frequencies were as follows:

Table 6.5
Dropouts after Randomization

	Total Number	(%)
Completed Study	149	(72.3)
Unacceptable diaries/dosage	20	(8.7)
Failed to return diaries/dosecards	37	(18.0)
	206	100.0

6.4.3 Comparability of treatment groups

During the period of the study, the number of subjects eligible for inclusion in the study fell from 206 to 149, based on the criteria for eligibility already described (of those ineligible, 20 returned inadequate diaries, while 37 dropped out of the study). It is therefore important to ensure not only that groups were comparable at randomization, but that there was no selectiveness amongst dropouts which changed the relative composition of the treatment groups during the study. Groups were compared for age, sex distribution, socio-economic, medical history and smoking variables (table 5.5). The mean age of children in the supplemented group was significantly lower at randomization (supplemented=4.6yrs, placebo=5.0yrs, p=.009) but this difference was smaller and non-significant at the conclusion of the study. The total study population contained initially more males than females (59%), as would be expected in a population selected in this way. The proportion of males was 62% and 57% in supplemented and placebo groups, respectively. At the end of the study, this was reduced to 52% and 60% respectively, indicating that, overall, a higher proportion of males than females had dropped out.

Table 6.6
Characteristics of Supplemented and Placebo Groups at Randomization

	Supplemented	Placebo
N	104	102
Age mths (SEM)	54.1(1.4)	60.2(1.8)*
Sex (%M)	62	57
>6 episodes URI in past 12 months	52	54
>3 episodes LRI in past 12 months	50	42
History of asthma	41	44
History of bronchitis	55	55
Fathers occupation:		
class: 4 - 6	77	69
7 - 9	14	23
Mothers education:		
primary only	3	8
high school	75	77
Home tenure: rented	30	33
Trust home	44	42
Current smokers at home	63	66
Smokers during 1st 12 months	70	75

URI = sore throat, runny nose, cold-like symptoms
LRI = pneumonia, bronchitis, wheeze, persistent cough
*p = 0.009

Table 6.7

Characteristics of Supplemented and Placebo Groups at the conclusion of the study after exclusion of those failing to meet criteria for compliance

	Supplemented	Placebo
N	79	70
Age mths (SEM)	56.2(1.6)	60.3(2.2)*
Sex (%M)	52	60
>6 episodes URI in past 12 months	47	57
>3 episodes LRI in past 12 months	47	44
History of asthma	43	44
History of bronchitis	57	54
Fathers occupation:		
class: 4 - 6	78	73
7 - 9	12	23
Mothers education:		
primary only	1	7
high school	72	69
Home tenure: rented	30	34
Trust home	43	43
Current smokers at home	64	67
Smokers during 1st 12 months	68	74

URI = sore throat, runny nose, cold-like symptoms

LRI = pneumonia, bronchitis, wheeze, persistent cough

* not significant

6.4.4 Characteristics of the population

The population, selected in the way described in sections 6.3 and 6.4 appeared to be unusual in several respects (tables 6.5 and 6.6). The study population was predominantly male (59%). A majority of families (64%) reported current smokers in the home, while 70% reported at least one parent smoking at home during the first twelve months of life of the child. This was considerably higher than the smoking history found in the first study, where 55% of families had current smokers and 44% had smokers during the first 12 months of life. Measures of socio-economic status indicated a preponderance of low socioeconomic groups. The population comprised 77% father's occupation classes 4-6 (similar to the previous study), while 74% of mothers had primary school education only. Housing Trust accommodation was reported by 43% of parents (these data were not able to be compared between the two groups).

Perhaps the most significant characteristic of the population was the heavy burden of illness reported at the initial interview. Thus 50% of children were reported to have had 6 or more episodes of sore throat, runny nose or cold-like symptoms during the preceding 12 months, while 45% had had three or more episodes of lower respiratory illness (pneumonia, bronchitis, wheeze and persistent cough) during that period.

As reported in other studies (section 6.1), a high proportion of children reported a history of asthma (43%), while yet more (54%) reported either history of asthma or wheezy bronchitis. Not many parents (18%) reported their children as currently taking asthma medication, although a

considerable proportion (45%) reported signs of current illness (wheezing apart from colds). Almost three quarters of the population reported wheezing with colds. These figures are consistent with a considerable amount of untreated asthma and undiagnosed bronchial hyperreactivity in this population.

Children who did not report a history of asthma or wheezy bronchitis (46%) tended to have a lower frequency of all respiratory symptoms: only 30% had 6 or more episodes of upper respiratory illness in the preceding 12 months, compared with 55% in the asthmatics group. Only 11.5% had more than three episodes of LRI in the preceding 12 months compared with 53% in the asthmatic group. As expected, conditions which could be related to asthma were more frequent in the hyperreactive group.

The non-asthmatic group had a similar mean age and socio-economic status when considered by father's occupation and education of mother, although home tenure variables indicate a smaller proportion of rented (23%) and housing trust homes (27%) in this group. Smoking history of parents was the same in both groups, however medical history showed a higher frequency of bronchitis, pneumonia, asthma and hay fever in the asthmatic group.

The mean number of respiratory episodes (12.3) was higher than that recorded in the previous study (7.2). The total days of nasal symptoms was similar in the two studies (58.5, 58.0, first and second respectively). However days of deep chest cough and wheezing were higher in the second study (32.0) than the first (14.0).

Thus, to summarize, there is a high prevalence of both prospective and retrospective respiratory illness in the population and this appears to be associated mainly with children who report a history of asthma. Such children do not appear to differ in social and environmental variables, but do have a higher frequency of allergic conditions and parental history of LRI.

Table 6.8
Comparison of First and Second Study Populations

Variable	Study 1	Study 2
N	127	206
Age months (SD)	38.7 (12.4)	57.1 (16.7)
Sex (%M)	50	59
Fathers Occupation		
1 - 3	17	8
4 - 6	77	72
Unemployed	7	20
Father's Education		
Matric	14	17
High School only	45	65
Mother's Occupation		
Working	25	35
Not working	75	65
Mother's Education		
Matric	9	8
High School only	71	74

Table 6.9

Mean Daily Intake of Major Nutrients (SD)

	Study 1	Study 2	RDA (Aus)	Stuckey	Magarey
N	122	149	-	120	178
Age (months)	38.7(12)	57 (17)	36-84	67	48
Mjoule	5.6 (1.3)	590(1.4)	7.2	7.7	5.9
(Kjoule/Kg)	376(108)	324(184	389	-	320
Protein (gm)	44 (14)	45 (11)	25-51	59	50
(gm/Kg)	3.0 (1.1)	2.5 (0.7)	1.3-2.8	-	2.8
Total Fat (gm)	54 (17)	61 (19)	-	79	57
Total CHO (gm)	173	182	-	230	187
Iron (mg)	6.6 (2.2)	7.1 (2.4)	7	10.6	7.3
VitC (mg)	81 (61)	46 (40)	30	90	77
Total VitA (ug re)	670(634)	612(367)	350	764	-
(ug re/Kg)	46 (39)	34 (22)	19	-	-
Carotenoids (ugre)	387(350)	297(272)	-	-	-
Height (cm)	95.8(9.7)	106.8(9.9)	-	-	
Weight (Kg)	15.4(3.6)	18.8(4.1)	18.5	-	-

6.4.5 Dietary intake of vitamin A

6.4.5.1 General Adequacy of the Diet

Dietary intakes of major nutrients in the two studies and other surveys of comparably aged children and the RDA are shown in table 6.9. Estimates are based on data from 3-day diet diaries in both studies reported here. Energy intakes in the second study group are low compared with the previous group, when the difference in mean age is taken into account.

They are also low in absolute terms, in comparison with the results of Stuckey and the RDA for this age group. Magarey and Boulton (1984) also found low energy intakes in Australian children and speculated that it may represent low energy expenditure due to restricted recreational activity. Protein intakes were similar to other studies and adequate in terms of the RDA.

Mean growth indices for males and females are shown in table 6.10.

Table 6.10
Growth Indices by Sex

	Males (Percentile)	Females (Percentile)
N	122	84
Age (months)	60	53
Height	108.3	102.9
(SD)	(10.1)	(10.1)
Weight	19.4	17.3
(SD)	(4.4)	(4.0)

Growth in females was adequate, corresponding with the 50-90 percentile, however that in males, for both height and weight was in the 25-50 percentile. Mean energy intake did not differ between males and females (324 and 323 Kjoule/Kg. day respectively). The poorer growth performance of males may therefore represent their higher frequency of illness.

6.4.5.2 Intake and Sources of Vitamin A

Total dietary intake of vitamin A was above both the Australian RDA of 350ug/day and the US RDA of 500ug/day. It was lower in absolute terms than the first study, despite the difference in mean age. Intake of carotenoids was lower in the second study (15.8ug re/kg) compared with the first (25.13ug/kg). Carotenoids represented 49% of total dietary vitamin A intake in the second study compared with 54% in the first group.

Table 6.11 shows the contribution of the supplement to overall vitamin A intake. The supplement contribution is calculated from the number of doses actually received by each child (determined from dose cards) times the amount of vitamin A present in the supplement at the time (determined by stability testing). Overall dietary intake of vitamin A refers to both retinol (preformed vitamin A) and ß-carotene (provitamin A) in the diet.

Table 6.11
Dietary and Supplementary Intake of Vitamin A (ug re/day)

	Supplement	Placebo
N	79	70
Total Dietary vitamin A (diary method)	570 (320)	661 (411)
Dietary + Supplementary A (diary method)	1093 (320)	661 (411)
Total Dietary vitamin A (frequency method)	1596 (990)	1265 (813)
Dietary + Supplementary A (frequency method)	2120 (989)	1265 (813)
Supplementary vitamin A	523	000

1. Diet Diary method 2. Diet frequency method

Total dietary intake of vitamin A was similar in supplement and placebo groups. The supplemented group received an additional mean daily intake of 523 ug re. This is considerably less than the 600ug/day initially targetted. Compliance with the dosage schedule was good - 68% of children (102) received the total number of doses, while 92% children (138) received 46/48 doses. The activity of the supplement was less than expected however, delivering 530ug/dose on a daily basis. This dose resulted in a treatment: placebo increase in mean total vitamin A intake of 1.6 times, based on diary dietary assessment and 1.7 times based on the frequency diary assessment. The difference in intake is not great in pharmacological terms, but is in accordance with change in intake

achievable by dietary manipulation.

6.4.5.3 Comparison of Dietary Intake of Vitamin A using Diary and Frequency Methods

Dietary intake of vitamin A was measured in two ways: by 3-day diet diary, and by a frequency method. Traditionally, a diary method has been thought to estimate absolute intake, and the frequency method only a relative estimate. However daily intake of vitamin A is highly variable - 1/2 carrot can supply the entire recommended daily allowance of a child and 1/4 cup liver supplies 10X the recommended daily allowance. Major sources of vitamin A are restricted in number, and many A-rich foods, eg, liver, carrot/vegetable soup and spinach may be eaten only once per month. Such infrequent intake is still beneficial, because vitamin A can be stored and mobilized during periods of dietary restriction. A method which averages intake over a long period is therefore more likely to reflect diet-related vitamin A status, than one which measures it over 3 days. The latter method would be expected to yield a much lower estimate of average daily intake, and this was observed in this study (table 6.12). Stuckey reported a similar intake of vitamin A when using a 2-day diet diary.

Correlation between the two different methods of estimating intakes was determined non-parametrically. Spearman's correlation coefficients between mid-study diary, and frequency estimates at the beginning and end of the study were 0.34 (p=0.000) and 0.21 (p=0.006) respectively. The measures were thus weakly but significantly correlated. Mean intakes and

ranges are given in table 6.12 and emphasize the variability achieved from use of different methods.

Table 6.12

Comparison of Dietary vitamin A Intake Estimates Using Diary and Frequency Methods (ugre/day)

	Mean	SD	Minimum	Maximum
TOTAL VITAMIN A				
Diary	612	367	80	2115
Frequency initial	1480	976	171	6391
" final	1440	922	7	4742
TOTAL ß-CAROTENE				
DIARY	297	270	21	1409
Frequency initial	1145	948	13	6226
" final	1071	843	1	4618

It can be seen that using both methods, some individuals were potentially at risk in terms of adequacy of intake of vitamin A, even though mean intakes for the whole population were well above recommended daily allowances.

Frequency estimates were made at the beginning and end of the study to determine whether intakes changed. This could well happen through increasing awareness of diet, due to the study itself. Vitamin A intake did

not change in the treatment group. In the placebo group, reported intake dropped (by 17%) over the period of the study. This difference was not significant, however

6.4.6 Distribution of Plasma Retinol in the Study Population

Plasma retinol was normally distributed with mean 39.18 (sem 0.872) ug/dl. Initial plasma retinol was correlated with final plasma retinol (r=0.55, p=0.000), and the distribution was similar in spread (SD1 10.7, SD2 12.0), however the mean decreased to 36.17 ug/dl. The difference was significant (T=3.25, p=0.001). A scatterplot of change in plasma retinol against initial plasma retinol showed that this drop was due mainly to reductions in initially high values, rather than an overall reduction in low and high values. This would suggest that the observed effect represents regression to the mean.

Mean plasma retinol in the second study was significantly below that of the first study (table 6.13) despite the younger age group of the first study, and its selection on the basis of frequent recent respiratory illness.

Table 6.13
Mean Plasma Retinol at the Beginning and Conclusion of Two Studies

	Initial			Final		
	N	mean	SEM	N	mean	SEM
Study 1	128	49.00	1.1	93	48.7	1.4
Study 2	176	39.15	0.9	153	36.7	0.7

Although the mean of 39 ug/dl is well above the level at which xerophthalmic changes commonly occur (<10ug/dl, WHO, 1974), the range of values observed (11.7 -73.9 ug/dl) were such that abnormalities due to marginal deficiency could be expected in a proportion of cases. Two published studies have examined the relationship between plasma retinol and marginal vitamin A deficiency at plasma levels of between 20 and 40 ug/dl. Flores (1984) measured relative dose response (RDR) in a group of mildly undernourished Brazilian children. This method assesses liver reserves of vitamin A by measuring the proportional increase in plasma retinol following a challenge dose of vitamin A (450 ugre). When adequate reserves are present, no increase occurred. At plasma retinol levels of less than 20 ug/dl, a positive response was observed in all children. At levels above this, progressively fewer children were positive, until at >40 ug/dl, only 3% responded (table 6.14). A similar relationship was found by Carney and Russel (1980) when studying dark adaptation in adults (vitamin A deficiency was secondary to liver disease, gastrointestinal disease and chronic alcoholism). Abnormalities occurred in similar

proportions of subjects above 20 ug/dl. Other dark adaptation studies contain results consistent with these observations (Fulton, 1982: Garrett-Laster 1984).

Table 6.14
Relationship between Plasma Retinol Levels and Marginal Vitamin A deficiency (from previous studies)

Plasma Vitamin A (ug re/dl)	Relative Dose Response* % Abnormal (N)	Dark Adaptation** % Abnormal (N)
>40	3 (39)	4 (27)
30-40	26 (19)	32 (19)
21-29	86 (21)	75 (12)
<20	100 (12)	67 (9)

* Flores et al, 1984.

** Carney and Russel, 1980

The relationship between plasma retinol level and the proportion of abnormal test results which is shown in table 6.15 was applied to the populations of study 1 and study 2 in order to determine the numbers of children potentially at risk of marginal deficiency. The results are shown in table 6.15

Table 6.15
Numbers of Children Predicted with Abnormal Relative Dose Response (RDR) and Dark Adaptation Responses

Plasma Vitamin A ug/dl	Study 1 N	RDR	DA	Study 2 N	RDR	DA
>40	83	2.5	3.3	70	2.1	2.8
30-40	38	9.9	12.1	71	18.5	22.7
21-29	5	4.3	3.8	27	23.2	20.0
<20	0	0.0	0.0	8	8.0	5.6
Total	126	20.7	19.2	176	51.8	50.3
% Abnormal		16.4	15.2		29.4	28.5

Thus, on this basis, 29% of children in study 2 would be expected to have an abnormal relative dose response (inadequate liver reserves of vitamin A) and the same proportion would be expected to show a deficit in dark adaptation. The assumption that the relationship in the two studies quoted can be extrapolated to the current studies may not necessarily hold. Different metholology of plasma vitamin A determination, for example, may alter the quantitative relationship. The HPLC method used in the current studies measures only bound vitamin A and not the unbound, postabsorptive form. The RDR and DA studies used spectrophotometric and fluorometric methods which measure total vitamin A. However the

difference between the two would not be expected to be large - less than 10% of vitamin A present in plasma is in the esterified form (Underwood, 1974).

Correlates of plasma retinol which could contribute to the difference in blood vitamin between the first and second trials were considered. Plasma retinol tends to increase with age (Underwood, 1974; Mejia, 1984) and thus age difference between the two trials could not account for the difference, but would have a reverse effect to that observed (plasma retinol was not correlated with age at all in the second study, and only weakly correlated with initial plasma retinol in the first ($r=0.16$, $p=0.03$)).

Socio-economic status has been correlated with plasma retinol in US studies (Owen et at, 1974; Underwood, 1984). A comparison of the variable Father's Occupation in the two populations indicated that the second study population comprised a lower socio-economic group. However correlation between father's occupation and plasma retinol within the study populations was weak, and only occurred with initial plasma retinol ($r=-0.13$, $p=0.11$: $r=0.18$, $p=0.18$ for study 1 and 2 respectively). It is unlikely that the difference between the two study groups in plasma retinol levels could be attributed to differences in socio-economic status.

Mean dietary intake measured by diet diary was 20% lower on the second study, however it was still well above the recommended daily allowance.

6.4.7 Plasma Retinol in Supplement and Placebo Groups

There was no significant difference in plasma retinol between the supplemented and placebo groups at the beginning of the study and none at the end of the study (table 6.16).

Table 6.16
Mean Plasma Retinol in Supplements and Placebo Groups at the Beginning and Conclusion of the Study

	Initial			Final		
	N	Mean	SEM	N	Mean	SEM
Placebo	56	37.0	1.4	62	35.5	0.9
Vitamin A	71	40.8	1.4	68	36.5	1.0

Mean plasma retinol, as stated above, decreased in the whole group over the period of the study, probably due to regression to the mean. The decrease occurred in both supplemented and placebo groups, however it was greater in supplemented group (3.6 ug/dl) than in the placebo (1.79 ug/dl). The change in plasma retinol in the supplemented group was not significantly different to that in the placebo group, leading to the conclusion that supplementation with vitamin A had no effect on plasma retinol values. This is consistent with four hypotheses:

1. The supplement was not taken
2. The supplement was taken but made no difference because :

(1) vitamin A levels were adequate.

(2) the dose was too small

3. Power of the study was not adequate to detect a difference in plasma retinol values
4. The supplement was taken but made no difference because vitamin A was not absorbed or mobilized.

Because of bottle checking and dosecard procedures, this result is unlikely to be due to large-scale non-compliance (hypothesis 1). It is more difficult to distinguish between the second two hypotheses. However if studies on relative dose response and dark adaptation can be extrapolated to this work, a large proportion of the population (29%) would be expected to have below optimal levels in the range 10-40 ug/dl and thus to respond to supplementation (Flores et at 1984). The size of the dose (the recommended daily allowance) is that expected to sustain optimal levels in children with normal uptake and mobilization of vitamin A.

The power of the study to detect a change of 20% in plasma retinol (a=.05) is 72%, however if a change occurred only in a sub-group comprising 29% of the population, the power would not have been adequate to detect it. Nevertheless a plot of change in plasma retinol versus initial retinol values showed no differential increase at lower blood retinol values. Plasma retinol did not increase even in those with initially low values.

In conclusion therefore, the failure to respond to supplementation may indicate that in this population, the blood vitamin A levels measured are

adequate. Alternatively it may indicate difficulty in utilizing additional dietary vitamin A in this group of children, because of reduced uptake or mobilization infection, or other conditions.

6.4.8 Pulmonary Function

Pulmonary function tests were performed to see if the range of lung function indices in the study population was normal, and to determine if a change in bronchial hyperreactivity could be detected with supplementation.

Results of lung function tests on 134 children at the initiation and conclusion of the study are shown in table 6.17. Forced vital capacity (FVC), forced expiratory volume in one second (FEV1), flow rate at 25% and 50%, vital capacity (V25 and V50), functional residual capacity and forced expiratory flow between 25% and 75% were within the normal range. Mean values for tests done one year later are similar.

Table 6.17
Mean Values of Pulmonary Function Indices at the Initiation and Conclusion of the Study (% Predicted)

	Initial	sem	Final	sem
FVC	98.5	5.6	95.7	1.2
FEV1	96.8	6.3	89.9	1.2
PF	87.9	1.5	88.0	1.6
V50	77.6	1.7	75.4	2.0
V25	84.8	6.0	76.5	2.5
FEF 25-75	74.4	1.9	82.5	1.9
VFRC	74.0	2.2	70.7	2.3

Bronchial hyperreactivity was determined by response to inhaled bronchodilator and to histamine challenge. Results for children who completed the study are shown in table 6.18. A positive response was taken as a 10% reduction in FEV1 after bronchodilator inhalation, or 20% reduction after histamine. At the beginning of the study, 24% of children were positive, while 45% were positive at either the beginning or end of the study and would suggest an even higher proportion if three or four determinations were made. Pullan and Hey (1982) also reported a 24% response to exercise challenge in a cross-sectional study (one determination) of 10 year-old children with a history of RSV infection in infancy.

The histamine challenge test required much more cooperation from children over a longer period of time (1hr), so relatively few of the study population were able to complete this. Of the children tested, 80% showed evidence of hyperreactivity on the first occasion, and 100% on either occasion (Table 6.18).

Table 6.18
Response to Bronchodilator and Histamine Challenge at the Initiation and Conclusion of the Study

	Bronchodilator % positive (N)		Histamine % positive (N)		Skin Test % positive (N)	
Initial	24	(75)	90	(19)	32	(143)
Final	32	(75)	80	(15)		
Initial or Final	45	(69)	100	(17)		

This high proportion is consistent with the retrospective information given by parents on the initial questionnaire. In the entire population, 80% of parents reported a history of either asthma, wheezy bronchitis, wheeze with colds or wheeze apart from colds (N=149). However the results of the histamine challenge should not be extrapolated to the whole population because of the highly selected nature of the sample tested.

A positive response to the skin test was taken as a wheal of >1mm diameter in response to 1 or more antigens. Of 143 children tested. 32% responded positively (Pullan and Hey, 1982, using stricter criteria.

reported 16% and 25% in children who had RSV in infancy and controls, respectively). Proportions of hyperreactive and skin test positive children were similar in treatment and placebo groups (table 6.19).

Table 6.19
Hyperreactivity and Atopy in Treatment and Placebo Groups

	BD Positive % (N)	Hist positive % (N)	Skin Test positive % (N)
Placebo	47 (32)	100 (5)	31 (65)
Treatment	43 (37	100 (12)	35 (78)

6.4.9 Respiratory Illness in Supplement and Placebo Groups

Over the study period of 12 months, the population experienced a mean total of 12.3 episodes of respiratory symptons (Table 6.20). Of these, 39% (4.8) were of probable infectious origin according to the symptom classification discussed in section 6.3.5. The preponderance of non-infectious symptoms reflects the high proportion of children reporting symptoms of chronic illness in the questionnaire. Days of runny nose and chest cough were dominant symptoms in the group with 50% of the population experiencing 246 days of runny nose and 50% of the population experiencing 41 days of chesty cough. Surprisingly, the median number of days of wheezing was only 1 (mean 12.8) and the median number of prescriptions of asthma medication was only 1. Thus the respiratory

burden of the population appears to have been mainly associated with upper and lower respiratory tract symptoms which were not consistent with either acute, recurrent infection or frank asthma. The distribution of all respiratory symptom and illness variables was skewed to the right (tables 6.21, 6.22). The summary variables episodes of probable, unclear and doubtful infections had similar but less skewed distributions with the exception of episodes of probable infections and total episodes which were approximately normally distributed.

Tables 6.20-6.22 show summary and symptom variables in supplemented and placebo groups after 12 months of follow-up. Of the 7 summary variable comparisons, 2 differences were found to be significant at the 5% level (Mann-Whitney test). In both of these highly intercorrelated variables (number of doctor visits and number of antibiotic prescriptions), the supplemented group reported more events than the unsupplemented group. Of the 7 symptom variable comparisons, days of sore throat were significantly lower in the supplemented group ($p<.05$, Mann-Whitney test), but the total number of episodes of sore throat was not significantly different. Because of the number of comparisons, one or two 'significant' comparisons could occur by chance. It seems reasonable to conclude, therefore, that there was no difference in respiratory illness experience in the two treatment groups.

Three asthma-related variables (months of asthma medication, and days and episodes of chesty cough and wheezy breathing) were found to be non-significantly lower in the treatment group. However the effect was not significant even when only children with a history of asthma were

compared, and the difference was not evident when medians rather than means were compared.

Table 6.20
Mean Episodes of Respiratory Illness in Supplemented and Placebo Groups: Summary Variables

	Placebo		Supplement		Statistical
	mean	sem	mean	sem	Significance
Probable Infections	4.71	0.40	4.85	0.32	ns
Unclear Infections	3.16	0.33	3.24	0.26	ns
Doubtful Infections	4.36	0.52	4.92	0.55	ns
Total	12.26	0.81	12.34	0.79	ns

Table 6.21
Respiratory Illness in Supplemented and Placebo Groups: Median Episodes and Interquartile Range

	Placebo		Supplemented		Statistical
	Median	IQ Range[1]	Median	IQ Range	Significnce
N	70		79		
		SUMMARY AND ILLNESS VARIABLES			
Probable Infections	4.0	2-6	4.0	3-7	ns
Unclear Infections	2.0	1-2	3.0	2.5	ns
Doubtful Infections	3.0	1-2	3.0	1-5	ns
Scripts of Antibiotics	0.0	0-1	2.0	1-3	p=.026 MW[2]
Months of Asthma Med.	0.0	0-1	0.0	0	ns
Doctor Visits	2.0	0-2	2.0	1-5	p=.049 MW
		SYMPTOM VARIABLES			
Sneezing	2.0	0-2	2.0	1-6	ns
Runny Nose	7.5	3-12	7.0	4-11	ns
Sore Throat	1.0	0-1	3.0	2-6	p=.046 MW
Chesty cough	3.0	2-6	3.0	2-6	ns
Ear Pain	1.0	0-3	1.9	0-2	ns
Wheezing	1.0	0-3	0.0	0-3	ns
Fever	2.0	1-4	2.0	1-3	ns

1 inter-quartile range

2 Mann-Whitney test

Table 6.22
Respiratory Illness in Supplemented and Placebo Groups: Median Days of Symptoms and Interquartile (IQ) Range

	Placebo		Supplemented		Statistical
	Median	IQ Range[1]	Median	IQ Range	Significance
N	70		79		
		SYMPTOM VARIABLES			
Sneezing	5.0	0-14	5.0	2-19	ns[2]
Runny Nose	32.0	14-84	35.0	17-88	ns
Sore Throat	3.5	1-8	5.0	2-14	ns
Chesty Cough	18.0	9-43	21.0	8-40	ns
Ear Pain	2.0	0-8	0.0	0-7	ns
Wheezing	1.0	0-7	0.0	0-8	ns
Fever	4.0	1-8	4.0	1-12	ns

1 Inter-quartile range
2 Mann-Whitney Test

6.4.9.1 Relationship Between Plasma Retinol Levels and Respiratory Illness in Supplement and Placebo Groups

We have shown that supplementation with vitamin A did not change episodes and days of respiratory symptoms, and also that plasma retinol levels in the population were relatively low. If these low levels were contributors to the amount of respiratory illness suffered, then some correlation between initial plasma retinol levels and subsequent illness would be expected, even if only at the lower end of the plasma retinol range. An association between illness and plasma retinol at the end of the study could be a result of illness itself. A positive correlation could indicate

greater utilization and then mobilization of vitamin A, associated with more illness. A negative correlation may mean inability of vitamin A reserves to sustain plasma retinol levels in the face of increased demand. A negative correlation could also reflect the short term reversible drop in plasma retinol which occurs during febrile illness.

With these possibilities in mind, initial, final levels and change in plasma retinol were tested for correlations with respiratory outcome variables in those children with values of plasma retinol <40ug/dl. Results are shown in table 6.23. Episodes of probably infection was significantly correlated with plasma retinol ($r = -0.20$, $p<0.05$) for range less than 40ug/dl as was days and episodes of runny nose ($r=-0.3$, $p=0.01$). However for each of initial, final and change in plasma levels, there were 6 comparisons. In order to allow for the increase in Type 1 error with multiple comparisons, a significance level of $a=0.05$ is more appropriate, giving a family wise Type 1 error role of 0.03 (Bonferroni test). Using this significance level none of the correlation of respiratory outcome variables with plasma retinol level were statistically significant.

Table 6.23
Correlation Between Plasma Retinol (<40 Ug/Dl) and Respiratory Variables

Plasma Retinol	Probable Infections	Unclear Infections	Doubtful Infections	Scripts Asthma	Doctor Visits	Hospital Visits
Initial	-0.20*	-0.12	-0.15	0.00	-0.03	-0.11
	(.048)	(0.15)	(0.11)		(0.40)	(0.16)
Final	0.03	-0.06	-0.02	0.10	-0.03	-0.15
	(0.37	(0.28)	(0.49)	(0.16)	(0.30)	(0.08)
Change	-0.16	0.21	0.08	-0.69	-0.04	-0.16
	(0.16)	(0.10)	(0.32)	(0.32)	(0.40)	(0.16)

Pearson's correlation coefficient
(statistical significance)

6.4.10 Multivariate Analysis of Medical, Social and Environmental Factors

Analysis of medical history, socio-economic and home environmental factors was undertaken both to control for confounding factors and to determine the relative contribution of factors other than vitamin A status to respiratory morbidity. The dietary vitamin A variables used were total vitamin A intake (ALLA), supplementary vitamin A (SUPPRET), and the dichotomous treatment variable (MED). The medical, social and environmental factors (Table 6.24) were derived from a questionnaire administered at the beginning of the study. Brochial hyperreactivity

variables were derived as described earlier from lung function measures at the beginning and end of the study. The strategy used in the analysis differed from that in the first study, in that, because of the larger number of variables, analysis was done in two stages. In the first stage, bivariate correlations between the variables described above and two dependent respiratory symptom variables were examined. In the second stage, those variables significantly associated with the outcome variables in bivariate analysis were entered into a multiple regression model.

The dependent variables examined in this way were total episodes of respiratory illness and episodes of probable respiratory infection. Both dependent variables were approximately normally distributed. Bivariate analysis of continuous variables was done using Pearson's Correlation Coefficient, and of categorical variables using one way analysis of variance. Analysis at this stage was on the entire, unselected study population. Variables tested in this way are shown in Table 6.24, results of the analysis are shown in Table 6.25, 6.26.

Table 6.24
Variables included in Bivariate Analysis of Social & Environmental Factors with Respiratory Outcome Variables

Variable	Description	Type
1. Medical History Model		
BRON	History of Bronchitis	d
PNEU	History of pneumonia	d
MEAS	History of measles	d
SINUS	History of sinus trouble	d
WHO	History of whooping cough	d
CROUP	History of croup	d
ECZEMA	History of eczema	d
RASH	History of hives, allergy rashes	d
COFHIS	History of cough lasting 3 months	d
WHZBRON	History of wheezy bronchitis	d
ASTHMA	History of asthma	d
AMED	Asthma medication taken for > 1 month	d
CURMED	Asthma medication taken currently	d
HAY	History of Hay Fever	d
EARINF	Frequent ear infections, 0-2 yrs	d
TUBES	Ever required ear tubes	d
TONSAD	Ever had operation for tonsils/adenoids	d
URI	Episodes of URI in past 12 months	o
LRI	Episodes of LRI in past 12 months	o
WKSPREM	Weeks premature	r
BRTHWT	Birthweight	r
BRSTMTH	Months breastfed	r
AGEMTHS	Age at entry to study	r

2. Family History Model

MOTBRON	Maternal history of bronchitis	d
MOTPNEU	Maternal history of pneumonia	d
MOTASTH	Maternal history of asthma	d
MOTHAY	Maternal history of hayfever	d
FATBRON	Paternal history of bronchitis	d
FATPNEU	Paternal history of pneumonia	d
FATASTH	Paternal history of asthma	d
FATHAY	Paternal history of hay fever	d

3. Home Environment Model

SMOKNOW	Smokers at home currently (beginning)	d
SMOKEND	Smokers at home currently (end)	d
SMOKPREV	Smokers at home, first year of life	d
HOMCON	Construction type of house	n
INSUL	Home insulation	o
AIRCON	Airconditioning	d
AIRTYP	Type (Evaporative or refrig.)	n
COOK	Type of cooker	n
HEAT	Home Heating	d
GASK	Gas heating or cooking	d
SHARE	Shared bedroom	o
ROOMS	No. of rooms in home	o

4. Socio-Economic Model

MOTED	Highest maternal education level	o
FATED	Highest paternal education level	o
MOTOCC	Maternal occupation (7 pt scale)	o
FATOCC	Paternal occupation (7 pt scale)	o
HOMTEN	Home tenure	n
TRUST	Housing trust home	o
ADLTS	No. of adults living at home	r

5. Hyperreactivity Model

BREACT1	Response to bronchodilator, beginning	d
BREACT2	Response to bronchodilator, end	d
BREACT3	Response to BD, beginning or end	d
REACTQ	Hyperreactivity based on questionnaire (History of asthma, wheezy bron., wheeze with colds or apart from colds)	d
SK	Response to skin allergen test	d

Variable type: d=dichotomous, n=nominal, o=ordinal, i=interval, r=ratio

Table 6.25
Association between Social, Medical History and Environmental Factors and Episodes of Respiratory Illness: Bivariate Analysis of Dichotomous Variables

Variable		Mean	SD	N	F	P
1. Total Episodes of Respiratory Infection						
History of:						
Croup	No	10.8	6.9	107	4.9	0.028
	Yes	13.2	6.4.	68		
Eczema	No	10.7	6.8	130	5.1	0.026
	Yes	13.3	6.1	47		
Rashes	No	10.9	6.3	123	4.9	0.028
	Yes	13.3	7.8	56		
Cough	No	11.0	6.8	140	4.6	0.033
	Yes	13.8	7.2	36		
Hayfever	No	10.4	6.2	109	8.3	0.005
	Yes	14.5	8.6	28		
Tubes	No	11.1	6.9	141	4.11	0.043
	Yes	13.5	6.6	41		
Skin test	No	10.9	6.0	127	4.7	0.031
positive	Yes	13.3	8.3	50		
2. Episodes of Probable Respiratory Infection						
History of:						
Sinus	No	4.1	2.9	135	5.4	0.02
	Yes	5.4	3.2	36		
Eczema	No	4.2	2.9	130	5.5	0.02
	Yes	5.4	3.6	47		
Rashes	No	4.1	2.9	123	5.9	0.015
	Yes	5.4	3.6	56		

		Mean	SD	N	F	P
Cough	No	4.1	2.9	140	8.3	0.005
	Yes	5.7	3.3	36		
Asthma	No	4.0	2.9	102	7.3	0.008
	Yes	5.3	3.4	76		
Asthma	No	4.1	2.7	59	4.3	0.04
medicat.	Yes	5.4	3.9	51		
Hayfever	No	4.0	2.8	109	7.9	0.006
	Yes	5.8	3.7	28		
Ear	No	3.9	2.7	104	9.5	0.002
infection	Yes	5.4	3.6	77		
Tubes	No	4.2	3.0	141	5.8	0.017
	Yes	5.6	3.6	41		
Sex (m)	No	4.6	7.2	69	0.1	0.77
	Yes	4.8	6.7	114		

Table 6.26
Factors and Episodes of Respiratory Illness: Bivariate Analysis of Continuous Variables

Variable	Pearson's Corr.	N	P
1. Total episodes of respiratory infection			
URI	0.21	181	0.003
LRI	0.19	181	0.005
2. Episodes of probable respiratory infection			
URI	0.26	181	0.000
LRI	0.29	181	0.000

Variables in only the medical history and hyperreactivity categories reached significance in the bivariate analysis. Two from the family history model were weakly significant (maternal bronchitis and maternal hayfever, $p<0.1$) and these were included in the multiple regression analysis. It is noteworthy that neither age nor sex were significantly correlated with respiratory episodes, when considered on their own, however because of their importance as correlates of respiratory illness in other studies, they were included in the final regression analysis. Of the socio-economic variables, only fathers occupation was weakly negatively correlated (a reversal of the traditional relationship). Lack of correlation between respiratory and socio-economic variables may be due to homogeneity in socio-economic status within the study population.

For the multiple regression analysis, a composite score was derived for respiratory illness history. This was the sum of the 16 unweighted dichotomous medical history variables shown in Table 6.24 (MEDHIS). History of cough lasting 3 months (a component of MEDHIS), LRI, URI, maternal history of bronchitis and skin test reactivity were also included in the regression, together with the vitamin A status variables already mentioned. Variables were introduced stepwise with criteria for inclusion being a change in R^2 having a significance of $p=0.05$. Natural logarithmic transformations of the dependent variables improved the fit of the model. Results are shown in Table 6.27.

Table 6.27
Variable Included in a Stepwise Multiple Regression Model with Episodes of Respiratory Symptoms as the Dependent Variables

Dependent Variable	Independent Predictor	B	R^2	T	P
Episodes of	COFHIS	0.31	0.057	2.7	0.008
Probable Infection	AGEMTHS	-0.01	0.051	-2.5	0.012
	Overall		0.108	(F) 7.0	0.001
Episodes	MEDHIS	0.03	0.045	2.3	0.02
all Symptoms	Overall		0.052	(F) 6.5	0.013

Of the variables entered into the regression, only history of cough and age for episodes of probable infection, and medical history score for episodes of all symptoms, proved to be predictors. Dietary vitamin A variables, including the supplementation variable, and plasma retinol did not contribute significantly to respiratory episodes experienced by children during the course of the study. Because it is possible that vitamin A status affects respiratory morbidity only when suboptimal (for example when plasma retinol is less than 30ug/dl), interaction terms for ALLA and plasma retinol were also entered into the model. However these did not contribute to overall variability.

It is noteworthy that only a small proportion of the variance (5-10%) was explained by the model (table 6.27) and thus its predictive and hence aetiological significance is limited. Nevertheless some conclusions can be

drawn. In general, variables which were indicators of prior respiratory illness or atopy were the strongest predictors of respiratory illness over the period of the study. This was confirmed in both bivariate and multivariate analysis. The lack of predictive power of the traditional risk factors probably reflects the uniqueness of the study population and to some extent its homogeneity, for example in social class. Selection of a population hospitalized in infancy and than the defacto selection which results in the reduction of potential membership from 643 to 206, produced a group of highly respiratory prone children - both on the basis of previous respiratory history (section 6.4.3) and prospectively measured symptoms (mean 12 episodes of respiratory symptoms in 12 months compared with 6.8 episodes reported for a slightly younger age group by Douglas and Miles, 1984). The major predictor of respiratory illness within the population therefore appears to be an underlying respiratory proneness or recurrent condition. The high proportion with bronchial hyperreactivity (assessed as 45% by response to bronchodilator, 83.5 % by history of wheeze with colds or asthma) would suggest this as a major contributor, however in the multiple regression analysis, these were less strong predictors than total respiratory illness history (MEDHIS for episodes of all symptoms and COFHIS for episodes of probable infection).

6.5 Discussion

The hypothesis tested in the second study was that oral vitamin A supplementation of children with a history of RSV positive bronchiolitis in infancy reduces episodes of respiratory symptoms. No benefit was

associated with supplementation, a result which is inconsistent with the results of the first study.

Much of the methodology is the same in the two studies, although the basis on which the populations were selected is different. Thus respiratory symptoms were measured by parent-completed diaries in both studies and the quality of recording was similar. The mean daily dose received was approximately the recommended daily allowance in both cases, although the dosage regimen differed: in the first study, doses were given daily over a period of 5 months, in the second, doses were given three times weekly over a period of a year. It could be postulated that daily dosing was more effective because newly absorbed vitamin A is used preferentially to the stored vitamin (Underwood, 1984). It is possible that the study population was not large enough to detect the 25% difference in episodes observed in the first study (retrospective power calculation indicated that a difference of 30-40% could be detected with a probability of 80% , a=0.05, in the second study). However the absence of an association between any indicator of vitamin A status (supplementation, total dietary intake or plasma retinol) and respiratory morbidity suggests that vitamin A status does not affect and is not affected by respiratory morbidity in this population.

The second study population differed from the first and from the general community in several respects. It was notable particularly for the high frequency of retrospective and prospective symptoms of chronic respiratory illness and bronchial hyperreactivity, also low energy intake, relatively poor growth indices and low socio-economic status. However the feature of most interest was the low mean plasma retinol recorded in the

group, both relative to children in the first study, and in comparison with other studies (section 6.4.5). Although the study was not designed to make comparisons of this type, the magnitude of the difference (20%), its stability over the 12 month period of the study, and the absence of other confounding covariates of plasma retinol which could explain the difference, raise the question of whether low plasma retinol levels are a characteristic of children who have been hospitalized with respiratory syncytial virus bronchiolitis in infancy.

6.6 Summary and Conclusions

6.6.1 A randomized, controlled trial of vitamin A supplementation was conduction in a population of children who had been hospitalized in infancy with respiratory syncytial virus-positive bronchiolitis.

6.6.2 The study population had a history of frequent respiratory illness and continued to experience an unusually high frequency of respiratory symptom episodes over the period of the trial. A high proportion of the population either reported symptoms of asthma or responded positively to tests of bronchial hyperreactivity.

6.6.3 Of 206 participants in the trial, 149 completed 12 months of supplementation and diary keeping and were included in the analysis. Ths supplemented group (N=79) was comparable to the placebo group (N=70) in age, sex, socio-economic, respiratory history and smoking variables.

6.6.4 Mean daily dietary vitamin A intake was 670ug re in the total population, well above the Recommended Daily Allowance (600 ug re), although minimum intakes indicated that some individuals had marginal intakes. Energy intakes were relatively low and growth indices were in the 25-50 percentile for males. Supplementation increased dietary intake by 85%.

6.6.5 Supplementation with vitamin A was not associated with a reduction in episodes or days of respiratory symptoms. Plasma retinol did not change in response to supplementation.

6.6.6 Plasma retinol levels were significantly lower in the second study population than the first and remained low for the duration of the trial. The range observed (11.7-73.9 ugre/dl) was low enough to include a proportion of children at risk of marginal vitamin A deficiency.

6.6.7 When all major risk factors (socio-economic, environmental, medical history, family medical history, dietary) were considered in bivariate and multivariate analysis, history of prior respiratory illness and atopy emerged as the major predictors of episodes of respiratory symptoms.

6.6.8 It is concluded that low-level vitamin A supplementation does not affect the respiratory morbidity of this population. However plasma retinol levels appear to be unusually low and unresponsive to changes in dietary intake. The origin of these low levels and the reason for the lack of response to oral supplementation remains to be determined.

Chapter 7

Summary and Conclusions

Respiratory illness covers a range of conditions which can be classified in many different ways: by anatomic site (tracheitis, bronchitis, pneumonia), by pathology (asthma, emphysema, empyema), by infectious agent (measles, tuberculosis, histoplasmosis) and by pattern of onset (acute, chronic, recurrent acute). Conditions with widely different aetiology have symptoms in common (cough, purulent nasal discharge, congestion, pulmonary function abnormalities) and most symptoms cannot be specifically associated with one disease. Two major aetiological categories of respiratory illness - infectious and noninfectious - display important interactions. Thus, the single most important precipitating factors for acute asthma has been reported to be respiratory viral infection (Carlsen, 1984). Chronic bronchitis sufferers often experience exacerbation of conditions as a result of viral infection (Crofton and Douglas, 1981). Chronic bronchitis itself is thought to result from a pattern of recurrent respiratory illness, often initiated with an acute infectious episode early in childhood (Samet, 1984). For these reasons, a symptom-based approach to the measurement of respiratory illness has been used in these studies. No attempt is made to distinguish episodes of infection for non-infectious events, with the exception of a method of symptom classification described in section 6.3.5. All conclusions from these studies therefore refer to conditions with a broad aetiological base, but with common functional, anatomical and clinical consequences.

The purpose of this work was to investigate whether vitamin A status is causally related to susceptibility to respiratory infection. In chapter 1, the public health significance of such an interaction in countries where vitamin A deficiency is endemic and morbidity and mortality due

respiratory infections is very high was discussed and the coincidence of peak prevalence of respiratory morbidity and vitamin A deficiency in the preschool years was noted. Studies by Sommer et al in Indonesia have examined the impact of vitamin A deficiency on respiratory morbidity and mortality and found a two- fold increase in respiratory morbidity in mildly vitamin A deficient children (Sommer et al, 1984).

Whether or not such an association contributes to morbidity and mortality in Western countries, where vitamin A deficiency is virtually unknown depends on the level of vitamin A deficiency at which an effect occurs and on the existence of subgroups in the community who are particularly at risk of vitamin A deficiency or susceptible to respiratory illness. Although the relationship which is of particularl interest in this thesis is the effect of vitamin A status on respiratory illness, the reverse relationship is well documented and is also important for two reasons. Firstly, it becomes necessary to chose a study design which enables the direction of the causal association to be determined, that is distinguishes cause and effect. Secondly, it establishes the possibility of a reciprocal relationship between marginal vitamin A status and respiratory illness which may extend and perpetuate the effects of a vitamin A- respiratory illness association.

The pilot study provided preliminary evidence, within a cross-sectional context, ofan association between vitamin A status and respiratory illness susceptibility in preschool-age children who experience frequent respiratory infections. This study introduced the concept of the respiratory-prone child , a subgroup in the community particularly

susceptible to respiratory infections, and an age at which vitamin A reserves are likely to be low. This cross-sectional study could not distinguish cause and effect in the relationship, however, and a different approach to the problem was taken at the next stage of the investigation.

In the ensuing study, a group of 'respiratory-prone' children was given a vitamin A supplement in a randomized, controlled study design, and the effect on respiratory symptoms was measured prospectively. This group of children were recruited from the community and their respiratory proneness had been defined in terms of frequency of respiratory symptoms during the preceding 3 months.

In the second study, respiratory proneness was found to be a stable characteristic of children, in that the frequency of respiratory symptoms was highly correlated from one 6 month period to the next. In the multivariate analyses of family history, medical, social and enviromental factors, the single strongest predictor of respiratory symptoms was symptom frequency during the preceding 6 months.

It was also concluded from that study that supplementation was associated with some reduction in episodes of symptom in these children, and that the individuals who appeared to benefit most from supplementation were those who had experienced lower respiratory illness earlier in childhood. Early history of chest illness, particularly in infancy has been extensively studied and well documented in the literature as being associated with respiratory symptoms and deficits in lung function, later in childhood and in adulthood. The theoretical basis for expecting an

association between vitamin A status in this group was strengthened by the fact that marginal vitamin A status modulates proliferation and differentiation of bronchiolar epithelial tissue. This tissue is also particularly susceptible to damage from respiratory viral infection early in life. It was decided, therefore , on the basis of some evidence of an effect of vitamin A supplementation in this group, and the plausible theoretical basis on which an effect could be expected, that it was worthwhile examining this group further. A study population in which 'lower respiratory illness' was more tightly defined was sought.

The third study concentrated, therefore on a relatively homogeneous group of 'respiratory prone' children, namely children who had been hospitalized in infancy with Respiratory Syncytial Virus-positive bronchiolitis. Again a randomized controlled trial of a low dose of vitamin A was tested and respiratory symptoms measured over a period of a year. This time, no supplementation benefit was observed - a result inconsistent with the results of the previous study. The supplementation regime had been equally effective in increasing dietary intake over the supplemented group (by a factor of 1.8 times in the first study and 1.7 times in the second) and other procedural differences between the two studies seemed inadequate to explain the difference in results (section 6.5). Both the 19% difference in episodes in the total population and the 25% difference in sub-group analysis were statistically significant at only the 5% level and no difference was observed with supplementation in total days of symptoms. These results are therefore at the limits of significance and it is possible that the difference observed was due to chance and does

not represent a real effect of supplementation. In the second randomized controlled trial, no difference was observed with supplementation in either total episodes or total days of symptoms, or episodes and days of symptoms considered individually. The power to detect a difference in episodes was not as great in the second study, as in the first (section 6.5), and it is possible that a difference of 25% was not detected in this study by chance. However no association between total dietary vitamin A intake and respiratory episodes could be found in either study. It seems reasonable to conclude that dietary vitamin A intake (and hence supplementation) was not a significant determinant of respiratory morbidity in the two populations.

The second trial population was very different to the preceding one. It was not a community-based group, but rather selected on the basis of an early episode of hospitalization. It proved to be a group with high frequency of upper and lower respiratory illness, and hyperreactive respiratory illness in particular. Moreover, when their vitamin A status was examined, plasma retinol was 20% lower than in the previous study - an unusual finding in a Western population and when dietary intake is adequate. Use of the community based group of children in the first study as a comparison poses problems since it is not comparable on the basis of age, sex, socio-economic status and illness history. Most of these differences would have had a conservative effect, however and thus comparison with a group of children which was not only comparable with respect to major confounding factors, but also experienced normal respiratory morbidity (i.e. not respiratory prone) may have produced an even bigger difference. It seems reasonable to conclude that plasma retinol was lower than

normally expected in the group of children chosen on the basis of lower respiratory infection in infancy.

Plasma retinol did not change with supplementation. This could be due to wholesale non-compliance, or to too low a doseage, but such an explanation would not account for low inital values in the face of adequate dietary intake. An alternative explanation is that dietary vitamin A is not readily utilized in these children due to problems of absorption, mobilization or metabolic changes secondary to recurrent infection or chronic illness (discussed in chapter 2) and thus an adequate diet, or vitamin A supplementation is ineffective in maintaining vitamin A status. Frequent episodes of illness may contribute to lower plasma retinol levels in this group. The lack of response to oral supplementation with vitamin A may explain why no change in respiratory morbidity occurred with supplementation.

The major ongoing questions relating to vitamin A status and its role in determining susceptibility to respiratory illness arise from the second study. If vitamin A status is low in this group of two to six year old children, and did not change over a twelve month period, when did it become low? Was it low at birth, and if so, could it have contributed to the original episode of respiratory illness which established a pattern of proneness in these children? A study which examines low neonatal vitamin A status as a risk factor for illness in the first year of life could well answer some of these questions. The importance of clarifying some of these issues is clear in the face of the major role of respiratory illness as a contributor to morbidity, both in the developed and developing world. The

establishment of a pattern of disease characteristic of the individual at and early age (respiratory proneness), and the unique position, if effective, that vitamin A could take as a preventive agent in the control of respiratory illness in the developed and developing world, suggest that further studies of the relationship between vitamin A status and susceptibility to respiratory illness would be well worthwhile.

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Appendix

Appendix 3.1

PROCEDURE FOR CHEEK CELL COLLECTION

BEFORE BREAKFAST ON MONDAY, WEDNESDAY AND FRIDAY MORNINGS:

1. Take 10ml of distilled water and rinse mouth out with gentle swishing. Discard washings.

2. Take 5ml of distilled water. Swish very vigorously around mouth for 10 seconds. Save washings in little pot.

3. Repeat step 2. three more times, making 4 rinses altogether.

The collected washings should look very cloudy - the cloudier the better! If they are not cloudy, squish more vigorously.

4. Wrap pot in foil and place it in the fridge, immediately. When you are ready to leave home, carry the pot in the insulated container provided and place it in the cool box on Carole's desk in the dept of CM. Keep your stubby holder for the next collection.

If you have any questions, or for any reason cannot get your collection in to the Department, please ring Carole on 79 8815.

MANY THANKS.

Appendix 5.1

NEWSLETTER

To Parents Participating in the Respiratory Vaccine Project

Dear Parents,

Enclosed is your diary for the six months after you have completed the present one. For some of you that won't be until September or October and you should not begin on the new one until you have completed the last page of the present one. When the present one is complete, it would be appreciated if you could drop it in to the receptionist at whichever of the following places is most convenient - Ingle Farm Community Health Centre, Florey, 105 Beovich Road, Ingle Farm or the St. Agnes Health Centre.

We are maintaining a continuously updated computer file on each child in the project and as the diaries come back, we are entering all of the information that you keep, together with information about your child's visits to the doctor on the computer. After the results of all completed diaries are entered on the computer in December, we plan to programme the computer with the vaccine code (remaining completely unaware ourselves about which preparation each child has received) in order to ascertain whether any clear general trends are emerging. This will help us to decide how long the study needs to continue in order to help make national policy decisions about the vaccine. We will keep you fully informed as information becomes available but would not be expecting to break the code either to you or to ourselves, about which child has received which injection, before the end of 1982.

You will be interested to hear that the study is being extended to include 500 aboriginal children in the Alice Springs area where ear infections and chest infections are an enormous problem. We are also doing a detailed study of nose swabs in 400 of the 1280 Adelaide children participating in the project in an effort to understand better the behaviour of the pneumococcal germ among Adelaide children.

Another matter of great interest to us at present is the question of how diet and vitamins may be related to chest and ear infections. All of you have already helped considerably in answering questions on this matter and your help has given us some important leads. It seems entirely possible that some foods or vitamins are particularly effective in increasing children's natural defence mechanisms against cold germs. We are currently very interested in some components of fruit and vegetables and would like to hear, especially from mothers whose children have had more than 15 days of cough or more than 3 separate episodes of runny nose in the last three months, who would be interested in helping us further elucidate this question by some extra record keeping. A form about this is attached for you to return if you are willing to help us to work further on this question.

This project has attracted some international interest and we are about to have a visit from a Chinese doctor who is coming to work with us for some weeks and who is planning research work of a similar kind to our own in a commune just outside Peking. We hope that Dr. Gao will be able to accompany

our nurses on some of their visits, especially the children who are participating in the nose swab study. We appreciate your continuing co-operation.

Yours sincerely,

R.M. DOUGLAS

Appendix 5.2

QUESTIONS AND ANSWERS CONCERNING THE NUTRITION AND RESPIRATORY ILLNESS STUDY

Q. What is the Purpose of this study?

A. We are attempting to find out whether some particular dietary constituents make a difference to childrens ability to withstand infections of the respiratory tract. Our preliminary experience suggests that children with lots of respiratory infections may have lower levels of one of the important vitamins than children who don't. This is not to say that their diets are deficient in this vitamin or that their blood levels of this vitamin are inadequate. In fact, our experience to this point suggests, as do other Australian studies, that most Adelaide children have what is agreed to be an adequate amount of this vitamin in their diet. The question has arisen whether children who are having recurrent episodes could benefit from additional amounts of this vitamin over and above the internationally agreed intake. And that is what we are trying to put to the test in this study. We have therefore selected children from the respiratory vaccine study who are known to be having lots of respiratory infections and are offering half of them a low level supplementary dose of this vitamin to see whether it makes a difference to their experience of respiratory infections in the next 12 months. If it does turn out in this study that children receiving additional supplements of this vitamin have a reduced amount of respiratory sickness, that will be a very important finding not only for Australian children but also overseas and may lead to some revisions in vitamin policy.

Q. What is expected of me in this additional part of the respiratory study?

A. We would want you to fill out some reasonably comprehensive forms concerning your child's diet, home environment and family history of disease. This is to enable us to get an up-to-date idea of some of the issues we explored with you in an earlier questionnaire and to give us more detail on some of these issues. We would ask you to give your child a dose of pleasantly flavoured syrup three times a week over the next six months. Half of the bottles of syrup that we are distributing to children in the study will contain the vitamin supplement and the other half will not. Neither the doctors, nurses or yourselves will know which these are until the end of the study. We would ask that you continue the normal dietary pattern for your child. If you are willing, we will make a finger prick blood test to measure the level of the vitamin in your child's blood. If it is abnormally low we would let you know immediately and appropriate action would be taken. Some people in the study will be asked to keep a detailed record of everything their child eats over a period of four days.

Q. What are the risks of this procedure?

A. The vitamin in question is a normal constituent of our daily diet and the amounts included in the supplements are of proven safety. If however you are already giving vitamin supplements to your child it is important to tell the nurse so that we can then ensure that the overall dosage of this vitamin which your child is receiving is completely safe.

For the finger prick blood test we will only need to take two or three drops of blood from a brief finger prick and that will give us and you some information about the present blood levels of the vitamin in your child.

Q. What are the possible benefits to my child from this study?

A. If this nutrient has the effect we are seeking, then your child has a fifty percent chance of reducing his or her respiratory infections during the next 12 months. In either event his diet will be assessed for adequacy in this vitamin and if a finger prick specimen of blood is collected we will know the level of the vitamin in his blood which gives us a partial assessment of his nutrient status. We will also be paying particular attention to his growth pattern over the next 12 months and to the number and severity of his colds and respiratory infections.

Appendix 5.3

CONSENT FOR PARTICIPATION IN NUTRITION AND RESPIRATORY ILLNESS STUDY

I understand that the purpose of this project is to find out whether low dose supplements of vitamin A can change the likelihood of children developing respiratory infections.

I understand that as part of this project I will be expected to give my child a dose of a medicine which contains either the vitamin preparation or a pleasantly flavoured syrup three times weekly during the next 6 months.

I understand that neither I nor the people conducting the study will know which preparation my child has been receiving until the conclusion of the study and that I will be told at that time.

I agree to continue to maintain the respiratory events diary and also complet the special questionnaires which are part of this study.

I agree/do not agree to the collection of two fingerprick samples of blood which will be used to estimate whether my child is deficient in vitamin A and that if the blood levels are abnormally low I will be informed and appropriat action taken.

I understand that I am free to withdraw my child from the study at any time.

Parent _____ Nurse _____

Date _____

OFFICE USE ONLY

	NO	YES		
Vitamin Supplement				
	if yes	_____	_____	
		Brand	Amount Taken	
Appointment time _____		_____		

Appendix 5.4

PROTOCOL FOR NURSES: EXPLANATION OF THE QUESTIONNAIRE

The questionnaire has three main sections:

Part A and B	Diet
Part C	Family history and home environment
Part D	Number of contacts

Part A and B

Purpose: To determine the child's food pattern particularly with respect to vegetables and to estimate the average intake of vitamin A. The two sections form a double check.

Problems: Parents may find it hard to think of what an average day is. Emphasize that we want to find out what they usually eat at this time of year (theoretically one month prior to this date). If it is different on some days to others try to write down both kinds of foods, e.g., 1) lunch sandwich: vegemite or peanut paste, 2) vegetables, peas, beans or broccoli.

It is important to try to think of everything the child usually eats, including snacks, e.g. chips, sweets, finger in margarine pot, etc. Use comments section if it s hard to estimate particular foods.

In the weekend section (foods eaten on an average weekend) write in only those foods which are different to those normally eaten on a weekday.

Part C

Purpose: To obtain information on family history of ill-health, smoking and other factors.

Problems: If a parent feels a box is inadequate to describe a situation, they can write a comment beside the box.

Part D

Purpose: The purpose of this part is to estimate how many people, particularly children, the child comes in contact with in an average week. We are interested in his normal routine, i.e. things he does every week (or in a typical week). The other questions are designed to find out how many contacts, particularly with preschool-age children, the other members of the family may have.

Problems: There is no need to fill out every hour of the table of activities. Just block in (e.g. with a line) the appropriate time period for each activity.

Note: Question 2 is the important one in this section.

Visit [

NUTRITION AND RESPIRATORY ILLNESS STUDY

The purpose of this study is to find out more about ways in which we can reduce the amount of respiratory illness suffered by children with a history of frequent bouts of respiratory illness.

To do this, we must determine what your child normally eats, and various features about his family history and home environment. We must also gain some idea of the numbers and kinds of people he contacts each week.

We would appreciate your cooperation in filling out the following questionnaire. Part A and B relate to diet, part C to family history and home environment and part D relates to your child's activities.

Official Use Only

Name _____ 7

Date ___/___/___

Age (months) _____ 13 14

Height (cm) _____ 15 18

Weight (kg) _____ 19 22

Vitamin Supplements NO ☐

(or tonics, pills drops etc.) YES ☐ Brand _____ Frequency taken _____ 23

Number of Treatment Bottle ☐☐☐ Amount Taken _____ 24 26

PART C

FAMILY HISTORY AND HOME ENVIRONMENT

Official U

1. Please indicate where your child lived during the first and second years of his life if different from your current address.

1st year _____ SUBURB _____ STATE _____ COUNTRY 27 ☐

2nd year _____ SUBURB _____ STATE _____ COUNTRY

2. Has this child had any of the following illnesses: please tick the appropriate box.

	Before Age 2yrs	After Age 2yrs	
1) bronchitis, pneumonia, croup, whooping cough, persistent cough	☐	☐	28 ☐
2) asthma, hayfever, eczema or food allergy	☐	☐	29 ☐
3) persistent runny nose, sinus trouble, tonsillitis, frequent ear infections	☐	☐	30 ☐
4) measles (not German)	☐	☐	31 ☐

3. What are the ages of brothers and sisters living at home? Please tick the appropriate box.

	1	2	3	
1) 0-2 yrs	☐	☐	☐	32 ☐
2) 3-4 yrs	☐	☐	☐	33 ☐
3) 5-10 yrs	☐	☐	☐	34 ☐
4) 11-18 yrs	☐	☐	☐	35 ☐

4. What is the total number of adults living at home?

1) Less than 60 yrs of age ☐ 36 ☐

2) More than 60 yrs of age ☐ 37 ☐

5. What are the present occupations of adults living at home?

Father (or male adult) _____ 38 ☐

Mother (or female adult) _____ 39 ☐

Officia

6. How many years of full time education (or equivalent) have they completed?

Father (or male adult) ☐

Mother (or female adult) ☐

☐☐ 40 4.

☐☐ 42 4.

7. In the past twelve months, have adults living at home experienced sore throat, runny nose or other cold-like symptoms? Ring the number of episodes in the last year.

	Number of episodes in previous year						
1) Father	0	1	2	3	4	5	6 or more
2) Mother	0	1	2	3	4	5	6 or more
3) other adult	0	1	2	3	4	5	6 or more
4) other adult	0	1	2	3	4	5	6 or more

☐☐ 44 45

8. In the past twelve months, have this child's brothers and sisters experienced episodes of sore throat, runny nose or other cold-like symptoms? Ring the total number of episodes for brothers and sisters in each age group.

	Total No. of episodes						
1) 0-2 yrs	0	1	2	3	4	5	6 or more
2) 3-5 yrs	0	1	2	3	4	5	6 or more
3) 6-10 yrs	0	1	2	3	4	5	6 or more
4) 11-18 yrs	0	1	2	3	4	5	6 or more

☐☐ 46 47

9. Have any adults living at home experienced persistent cough or wheezing, or been told by a doctor they have bronchitis, pneumonia, emphysaema or other chest illness?

	No	Yes, this year	Yes, before this yr
1) Father	☐	☐	☐
2) Mother	☐	☐	☐
3) Other adults	☐	☐	☐

☐ 48

☐ 49

Office

10. Have any of this child's brothers and sisters experienced persistent cough or wheezing, or been told by a doctor they have bronchitis, pneumonia, croup or other chest illness?

Brother or sister	No	Yes, this yr	Yes, before this yr	
1	☐	☐	☐	☐ 50
2	☐	☐	☐	
3	☐	☐	☐	☐ 51
4	☐	☐	☐	

11. How many adults living at home now smoke regularly? (at least one cigarette per day or 28g of tobacco per month) ☐ ☐ 52

12. How many adults living at home smoke or smoked regularly during the first or second year of life of this child? ☐ ☐ 53

13. What method of home heating to do you use?

1) none ☐
2) electric, oil ☐
3) gas, gas & electric ☐
4) other (please specify) _____

☐ 54

14. What method of cooking do you use?

1) electric ☐
2) gas ☐
3) other (please specify) _____

☐ 55

15. Is your home air conditioned?

1) No ☐
2) Partial (1-2 rooms) ☐
3) Fully (all rooms) ☐

☐ 56

16. How many rooms (not counting bathrooms) are in your house? ☐ ☐ ☐ 57 58

Offic

17. If your child had the following conditions, please indicate what you would do.

	Regards it as a normal part of growing up	Apply home remedy or get something from the chemist	Consult a doctor	
Frequent runny nose				59
sore throat for 4 days				60
Frequent cough				61
often clears throat				62
frequently seems irritable and tired				63
oftens asks you to repeat words				64

Appendix 5.6

NAME _____ DOSE CARD _____ Bottle number

Dose: Three times per week. Place a tick for each dose given.

Week Beginning													
Week No.	1	2	3	4	5	6	7	8	9	10	11	12	13
Sunday													
Wednesday													
Friday													

Appendix 5.7

RESPIRATORY EVENTS DIARY

Definition of respiratory infection:

For the purposes of keeping this diary a bout of respiratory infection includes any episode of ill health in which the child experiences one or more of the following: ear infection, a "cold", sore throat, runny nose, flu, bronchitis and pneumonia".

Instructions for diary use:

1. For each month please fill in the name address and identification number of the child under study.

2. For any day of the month in which the child experiences the symptoms mentioned below, please cross in this date in the appropriate position on the table provided.

3. For each such day please indicate which of the following symptoms were present:

   (a) Ear pain/or fluid discharge from the ear

   (b) Sleep disturbance

   (c) A temperature

   (d) Excessive irritability

   (e) Nose and/or throat soreness/pain in sinus are/and/or runny nose

   (f) Hoarseness and/or cough

   (g) Deep chest coughs/wheezing and/or breathlessness

4. For each such day please indicate how seriously your child was affected by these symptoms using the following categories:

   1. Mild: no restriction to child's normal daily activities.

   2. Moderate: some distress to the child, whose normal daily activities are restricted.

   3. Severe: child is distressed, and quite unable to undertake normal daily activities.

NAME _____

ADDRESS _____

_____

TELEPHONE _____

IDENTIFICATION NUMBER

COMMENTS:

CHECKED BY

MONTH: Sept. YEAR: 1982

DAY	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
(a) ear pain/fluid discharge																															
(b) sleep distrubance																															
(c) temperature																															
(d) excessive irritability																															
(e) nose/throat/sinus																															
(f) hoarseness/cough																															
(g) deep chest cough/breathlessness																															
SEVERITY																															
MEDICAL ATTENTION																															

TAKEN

Appendix 5.9

MEDICAL CASENOTES STICKER

RESPIRATORY VACCINE STUDY (MEDICAL VISIT)

Date: ..... Cause of Visit: Acute Resp. Infection ☐ New ☐
Otitis Media ☐ Follow-up ☐
Other ☐

Clinical Diagnosis

Otitis Media:	right ☐	left ☐	both ☐
U.R.T.I.:	rhinitis ☐	sinusitis ☐	tonsillitis ☐
	pharyngitis ☐		
M.R.T.I.:	laryngitis/croup ☐	epiglottitis ☐	
L.R.T.I.:	bronchitis ☐	bronchiolitis ☐	pneumonia ☐

Medication

	Antibiotic ☐	Antihistamine ☐	Vasoconstrictor ☐
	Bronchodilator ☐	Other ☐	
Laboratory	N/P swab ☐	Ear aspirate ☐	Blood Culture ☐
	Other ☐		

Follow-up, Comments

Appendix 5.10

VACCINE TRIAL: RESPIRATORY EVENTS DIARY SIX MONTH SUMMARY SHEET

			Review Period
		IDENTIFICATION NUMBER	
		1-5	6
1.	Number of discrete episodes of probable Acute Respiratory Infection (A.R.I.).		7-9
2.	Total number of days on which one or more of symptoms (a), (b), (d) were noted.		10-12
3.	Total number of days on which one or more of the symptoms (e) to (g) were noted.		13-15
4.	Total number of days on which one or more of symptoms (a) to (g) were noted.		16-18
5.	Total number of days in which the following symptoms were noted.		
	(a) ear pain/fluid discharge from the ear		19-21
	(b) sleep disturbance		22-24
	(c) temperature		25-27
	(d) excessive irritability		28-30
	(e) nose and/or throat soreness/pain in sinus area and/or runny nose		31-33
	(f) hoarseness and/or cough		34-36
	(g) deep chest coughs/wheezing and/or breathlessness		37-39
6.	Number of separate courses on antibiotics (Totalling 3 or more days)		40-41
7.	Number of days on which antibiotics taken.		42-44
8.	Total number of days on which pharmaceutical preparations of all kinds were given (MIMS)		45-47
9.	Number of days on which Panadol and/or antihistamines were given.		48-50
10.	No. of days of distress and/or restriction to the child (2 or 3).		51-53
11.	No. of visits to the G.P. at the time of symptoms of possible Acute Respiratory Symptoms (a to g).		54-55
12.	Was the child free of symptoms on the last day? (YES-1, NO-2)		56
13.	How many patient days were documented.		57-59

14. Was the patient hospitalized during the review period?
    1. Yes, for respiratory symptoms
    2. Yes, for other reason
    3. No

60

15. If hospital: 1. A.C.H. 2. Modbury 3. Other

61

16 Total days hospital (respiratory illness).

62-63

17. Were all G.P. visits with one surgery?
    1. Yes - Ingle Farm Health Centre
    2. Yes - St. Agnes
    3. Yes - Florey
    4. Yes - other
    5. No

64

18. Quality of diary coding:
    1. Excellent 2. Some problems 3. Major Problems

65

Appendix 5.11

NUTRITION STUDY

DIET DIARY

This is the final section of the nutrition study. In this section, we ask you to keep a record of everything your child eats and drinks over a THREE DAY PERIOD.

First record the food itself and a description (e.g. cordial, lemon; a pie, apple) then the amount which is actually eaten. Use teaspoons, cups and tablespoons as measures, as before. We will provide you with a paper cup (150 ml) to use as a 1 cup measure.

We ask you to record foods eaten on three consecutive days, beginning on a Sunday, as soon as is convenient. Please return the forms either to the centre, or by mail.

Remember to write down everything you child eats including sweets, soft drinks and if possible - foods he helps himself to! Extra sheets are included in case you run out of space. You may find this time consuming and we thank you for your continuing patience and cooperation with the study.

NAME Jennifer Vanes

Food Record for DAY 1 Day of week Sunday

OFFICE USE ONLY

Day of Study 6 7 8 ID NUM 1 5 9 12

TIME OF EATING	Food Item & Description (brand names is possible)	COOKING METHOD	AMOUNT ACTUALLY EATEN
7am	Orange juice drink : Mr Juicy	–	½ cup.
	Weet bix	–	1 bix
	Milk on weetbix	–	½ cup
	Sugar	–	2 tspns
10 am	Twisties		1 packet
	apple		one quarter
	chocolate frog	–	1
12 noon	Cheese sandwich : Bread, wholemeal		2 slices
	Margarine, Flora		2 tablespns.
	Cheese, Kraft sliced	–	1 slice
	Fruit cake (shop-bought)	–	5cm x 3cm piece
3pm	carrot fresh	Raw	10cm stick
	peanuts, salted	–	½ cup
	orange juice, pure, Berri	–	1 cup
6pm	lamb, leg of	Roast	1 small slice
	peas, frozen	boiled	¼ cup
	carrots, fresh	boiled	1 tablespn
	potatoes	roast	1 small
	peaches, tinned	–	½ cup
	icecream, Streets vanilla	–	1 scoop

Food Code 13 16 Gms eaten 17 20

Menu Card 13 16 17 20

Menu Card 13 16 17 20

NAME _____

Food Record for DAY

Day of week _____

OFFICE USE ONLY

Day of Study 6 7 8

ID NUM 1 5 9 12

TIME OF EATING	Food Item & Description (brand names is possible)	COOKING METHOD	AMOUNT ACTUALLY EATEN	Food Code 13 16	Gms eaten 17 20
					24
					32
					40
					48
					56
					64
					72
				Meal Record 13 16	17 20
					24
					32
					40
					48
					56
					64
					72
				Meal Record 13 16	17 20
					24
					32
					40

Appendix 6.1

THE ADELAIDE CHILDREN'S HOSPITAL INC

NORTH ADELAIDE
SOUTH AUSTRALIA 5006
Telephone 267 XXXX 7234
Telex ACHOSP 89178
XXXXX

DR A J MARTIN, M.R.C.P. (U.K.)
Paediatric Pulmonologist

14th September, 1984

Dear Mr and Mrs _____,

Our records show that your child, _____ was admitted to hospital with a respiratory infection as an infant, and that this infection was associated witha germ called respiratory syncytial virus. Several studies have shown that children who have had this kind of illness have an increased amount of chest illness in following years. We have found that a particular vitamin supplement may be helpful in preventing this. This vitamin is a normal constituent of the diet, however our studies have indicated that children who tend to get chest infections may need more of it than other children. We will be studying the effectiveness of this nutrient in preventing respiratory illness over the next 12 months in a group of children who have had similar chest infections, and invite you to join this group.

If you would like to participate, a form is attached for you to return, and then our nurse will contact you to further explain what we are doing.

Yours sincerely,

A.James Martin .

Carole Pinnock
DEPARTMENT OF COMMUNITY MEDICINE.
UNIVERSITY OF ADELAIDE.

NUTRITION AND RESPIRATORY ILLNESS STUDY

NAME OF CHILD: _____

DATE OF BIRTH: _____

Please : 1. Contact me regarding the Nutrition and Respiratroy Illness Study.

2. Do not contact me regarding the Nutrition and Respiratory Illness Study.

(DELETE WHICHEVER APPLICABLE).

Signed _____
Parent or Guardian

Current Address _____
Street

_____
Suburb post code

Telephone Number: _____ (Daytime)

_____ (Evenings)

PLEASE RETURN IN THE ENCLOSED STAMPED ADDRESSED ENVELOPE WITHIN THE NEXT THREE DAYS.

- - - - - - - - -

Appendix 6.2

THE UNIVERSITY OF ADELAIDE

DEPARTMENT OF COMMUNITY MEDICINE

LEVEL 5, ADMINISTRATION BLOCK
ROYAL ADELAIDE HOSPITAL
ADELAIDE
SOUTH AUSTRALIA 5001
Telephone: (08) 223-0230
Telex UNIVAD AA 89141

NUTRITION AND RESPIRATORY ILLNESS STUDY

Dear Parent,

Thank you for your interest in the Nutrition and Respiratory Illness Study. I enclose an information sheet which outlines the purpose of the study and explains what it involves. We would be happy to answer any further questions you may have and our contact numbers are given on the sheet.

Our Study Nurse, Diane Markham will be contacting you shortly. If you would like to join the study, she will make an appointment for a clinical examination in November or December and we would like you to fill out the enclosed forms and bring them with you to this examination.

We appreciate your interest and hope you will join us in this worthwhile project.

Yours sincerely,

CAROLE PINNOCK,
Study Coordinator,
Nutrition & Respiratory Illness Study.

NUTRITION AND RESPIRATORY ILLNESS STUDY

INFORMATION SHEET FOR PARENTS

WHAT IS THE PURPOSE OF THE STUDY?

A number of recent studies have shown that some children who have had chest illnesses, such as respiratory syncytial virus infection, continue to have symptoms of chest illness more frequently, both as children and as adults. Recent evidence suggests that a vitamin which is normally present in the diet and known to have functions related to the respiratory tract, could be particularly important in respiratory illness. Small, additional amounts of this vitamin appear to be helpful in reducing respiratory symptoms in children who have had chest infections early in life. We have therefore selected a group of such children, and will offer half of them the vitamin supplement to see whether it can reduce the amount of respiratory illness they will have over the next 12 months.

WHAT WILL YOU HAVE TO DO?

We will ask you to give your child a teaspoon and a half of syrup each week over the next 12 months. This syrup may or may not contain the vitamin. It is necessary that neither the doctors, nurses or yourselves know whether or not it does until the study is finished, in order to be able to judge the effectiveness of the supplement fairly. During the period of medication, we would like you to record daily on a diary sheet whether your child has any respiratory symptoms. At the beginning and end of the study we will give your child an examination to find out how well his or her lungs are working. For these tests we measure how deeply your child breathes and how rapidly he can breathe out. We may perform a skin test to see if he is allergic. Two additional tests will tell us what you child's vitamin status is. The first test involves rinsing the mouth with water. The washings are collected and we measure the amount of vitamin in the cheek cells collected this way. This is a new method of assessment and we hope eventually, for some purposes, it will replace the second method, a conventional blood test. In the latter, we make a small prick in the finger and collect several drops of blood.

We will also ask you to fill out a questionnaire to give us some general information about your child, and one to tell us about the kinds of foods he likes to eat. We may ask you to keep a diary of the food your child eats over a three day period. All information collected in the study will remain confidential.

WHAT ARE THE RISKS?

The vitamin in question is normally present in, and essential for, a healthy diet. The amounts given are of proven safety. However, if given in amounts larger than in this study, the vitamin can be toxic. Although the effects are reversible, if your child is already receiving vitamin supplements it is important that you tell us. We can then ensure that the overall dose is completely safe.

WHAT ARE THE BENEFITS OF PARTICIPATING IN THE STUDY?

If the supplement is effective, your child has a one in two chance of reducing the amount of respiratory illness he will have next year. If he participates in the study we will be able to tell whether there are any significant problems as far as his lungs are concerned and whether his dietary intake and body levels of this vitamin are below levels considered necessary for good health. The possibility that a normal component of the diet may help those at risk of respiratory illness is an important finding as a change in diet is relatively easily accomplished. Respiratory syncytial virus infection is a common cause of illness in infancy and many people will stand to benefit if this supplement is proven effective. We hope you will join us in this project and look forward, at the conclusion of the study, to sharing with you our increased understanding of the problem.

WHO IS CONDUCTING THE STUDY?

The study is being conducted by the Pulmonary Department, Adelaide Children's Hospital in collaboration with the Department of Community Medicine, University of Adelaide. Interviews and examinations will be carried out at the Adelaide Children's Hospital by the study team. Dr. James Martin is the physician associated with the project, the study coordinator is Ms. Carole Pinnock from the Department of Community Medicine, University of Adelaide and Diane Markham is the nursing sister associated with the study.

This has been a short description of the study and we will be pleased to discuss any particular aspects with you in more detail. We would also like to make it clear that you are free to withdraw from the study at any time if you wish. If you have any further questions, we can be contacted at following numbers:

Dr. James Martin
Dept. of Pulmonary Medicine
Adelaide Children's Hospital
267-7234

Carole Pinnock (Study Coordinator)
Department of Community Medicine
University of Adelaide

Diane Markham (Study Nurse)
Department of Community Medicine
University of Adelaide

223-0230
ext. 6211

(a message can be left with the Secretary of the Dept. on ext. 5135 if there is no response)

Appendix 6.4

GENERAL QUESTIONNAIRE

Date: .....

1. Name of Child: .....

2. Home Address: ..... (Street)

..... (Suburb)

..... (Postcode)

3. Date of Birth: ..... Sex:

4. Does he/she regularly attend school? Yes/No

If 'yes' -

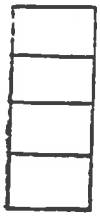
a) When did he/she first begin? ..... (Month) ..... (Year)

b) Give location of school: ..... (Suburb).... (Postcode)

ILLNESS HISTORY

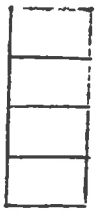
5. In the past 12 months, how many episodes of sore throat, runny nose or other cold-like symptoms has this child had?

a) 0 - 2
b) 3 - 5
c) 6 - 8
d) more than 8



6. In the past 12 months, how many episodes of chest illness has this child had (pneumonia, bronchitis, wheeze, persistent or productive cough) which kept him from his usual activities for three or more days?

a) 0
b) 1 - 2
c) 3 - 4
d) 5 or more



7. How old was this child at the time of his/her first chest illness?

a) 0 - 1 ☐
b 2 - 3 ☐
c) 4 - 5 ☐
d) 6 or more ☐

8. How many times has this child been hospitalized for chest illness? .....

9. In the past 12 months, how many weeks of kindergarten or school has this child missed due to colds, throat, ear or chest illnesses?

a) 1 or less ☐
b) 2 - 3 ☐
c) 4 - 8 ☐
d) greater than 8 ☐
e) does not apply ☐

10. Has a doctor ever said this child had any of the following illnesses?

	Yes	No	Don't Know
a) bronchitis			
b) bronchiolitis			
c) pneumonia			
d) measles (not german)			
e) sinus trouble			
f) whooping cough			
g) croup			
h) eczema			
i) hives or allergy rashes			
j) cough which lasted for 3 months or more			
k) wheezy bronchitis			
l) asthma			

If 'yes' to (k) or (l)

	Yes	No	Don't Know
m) Did he/she take medicine for periods exceeding one month in total?			
n) Is he/she currently taking medicine?			

11. Did this child have frequent ear infections (more than 2 a year):

	Yes	No	Don't Know
a) between age 0 and 2 yrs.			
b) between age 2 and 5 yrs.			
c) over age 5 yrs.			

12. Did this child ever require tubes in his/her ears to drain them?

13. Did this child ever have an operation on his/her tonsils or adenoids?

USUAL SYMPTOMS

14. Does this child usually have a cough:

	Yes	No
a) with colds?		
b) apart from colds?		

If 'yes' to (a) or (b) –

c) does he/she cough on most days (4 or more per week) for as much as 3 months of the year?

If 'yes' to (c)

d) for how many years has this occurred? .....

15. Does your child usually seem congested or cough up phlegm:

	Yes	No
a) with colds?		
b) apart from colds?		

If 'yes' to (a) or (b) –

c) does he/she seem congested or bring up phlegm on most days (4 or more per week) for as much as 3 months of the year?

If 'yes' to (c) –

d) for how many years has this occurred? .....

16. Does this child usually breathe –

a) through the nose

b) through the mouth

c) frequently through both

17. Does his/her breathing ever sound wheezy or whistling:

	Yes	No	Don't Know
a) when she has a cold			
b) occasionally, apart from colds			
c) most days or nights			

If yes to (b) or (c) -

d) at what age did the wheezing begin? .....months/years

18. Has this child ever had an episode of wheezing that caused him/her to be short of breath?

Yes	No	Don't Know

If yes:

a) how many such episodes has he/she had in the last 12 months? .....

b) has he/she ever required medicine for these episodes?

c) at what age did these episodes begin? .....

d) is his/her breathing completely normal between attacks?

19. Does this child ever get attacks of wheezing after playing hard or exercising?

20. When this child gets a chesty illness or wheezing, do you give him/her a medicine prescribed by a doctor?

If 'yes', does this include -

	Yes	No	Don't Know
a) an antibiotic (such as septrin, bactrim, amoxycillin)			
b) asthma medicine (such as ventolin, intal)			

BIRTH HISTORY

These questions relate to the birth of the child.

21. Was he/she delivered earlier than the due date?

Yes	No	Don't Know

If 'yes', how much earlier? ..... (wks.)

22. What was the birth weight of the child? ..... (kg.) or ..... (lbs.)

23. Was he/she kept in hospital after the mother went home?

Yes	No	

If 'yes' give reasons: .....

.....

24. Did the child experience difficulty breathing during the first few months of life?

Yes | No

If 'yes' describe what kind of difficulty. .....

.....

25. Was this child breastfed?

Yes | No

If 'yes', for how long? ..... (Mths)

FAMILY HISTORY

26. What are the present occupations of adults living at home?

a) mother (or female adult) .....

b) father (or male adult) .....

c) other .....

27. Please tick the highest educational qualification completed. This question applies only to adults living at home.

	Mother	Father
a) primary		
b) high school		
c) matriculation		
d) trade certificate or diploma		
e) university of CAE degree		
f) other (please specify)		

28. Do any persons living at home currently smoke cigarettes?

Yes | No

If 'yes' how many per day?

a) Mother ..... cigs/day

b) Father ..... cigs/day

c) Other ..... cigs/day

d) Other ..... cigs/day

29. Did any adults smoke regularly during the first year of life of this child?

Yes | No

If 'yes', how many per day?

a) Mother ..... cigs/day

b) Father ..... cigs/day

c) Other ..... cigs/day

d) Other ..... cigs/day

These questions apply to the child's natural parents.

30. Has the mother of this child ever been told by a doctor that she had –

	Yes	No	Don't Know
a) bronchitis			
b) pneumonia			
c) emphysema			
d) asthma			
e) hay fever			

31. Has the father of this child ever been told by a doctor that he had –

	Yes	No	Don't Know
a) bronchitis			
b) pneumonia			
c) emphysema			
d) asthma			
e) hay fever			

HOME ENVIRONMENT

32. How many other children are living at home?

a) 0 – 12 months .....
b) 1 – 2 years .....
c) 3 – 4 years .....
d) 5 – 10 years .....
e) 12 – 18 years .....

33. How many adults (over 18 yrs.) are living at home? .....

34. How many rooms (not counting bathrooms) are there in your house/flat? .....

35. Does this child share a bedroom?

a) no
b) with 1 person
c) with 2 persons
d) with 3 or more persons

36. Is your home heated?

a) not at all
b) some rooms
c) all rooms

37. What type of home heating do you use?
(Tick whichever apply.)

a) electricity
b) oil
c) gas
d) kerosene
e) open fire
f) other (specify) .....

38. What type of cooking facilities do you have?

a) electric
b) gas
c) other (specify) .....

39. Do you usually keep windows open in your house:

	Yes	No
a) in summer		
b) in winter		
c) in spring and autumn		

40. Do you use an extraction fan in your kitchen?

41. Is your home air conditioned (cooled)?

a) not at all
b) some rooms
c) all rooms

42. If yes to b) or c), what kind of air conditioner do you have?

a) evaporative cooler
b) refrigerant type
c) other (please specify)

.....

43. Is there anything which relates to your child's respiratory illness which you would like to comment on?

.....

.....

.....

.....

.....

Appendix 6.5

INFORMED CONSENT

I have received and read a copy of the information sheet and consent form. I understand that the purpose of the study is to learn whether a vitamin supplement can reduce the likelihood of children developing respiratory infections.

I understand that, as part of the study, I will be asked to give my child a weekly dose of syrup, and that this may or may not contain the nutrient of interest in the study.

I agree to maintain records of my child's respiratory symptoms as required, and will be willing to come with him to the Adelaide Children's Hospital for the tests and questionnaires described in the information sheet.

I understand that I will be informed promptly if any test results are abnormal and that all information collected in the study will be confidential.

I know that I may withdraw my child from the study at any time.

_____ _____

Signature (Nurse)

Signature (Parent)

EXCLUSION QUESTIONNAIRE

1. Does this child currently have any serious illnesses or other medical conditions?
   No____ Yes____ if yes, what are they?

   _____

   _____

2. Is he/she currently taking any medicines?
   No____ Yes____ if yes, what are they?

   _____

   _____

3. Has your child taken any vitamin supplements (pills, tonics, syrups) over the last 6 months?
   No____ Yes____ if yes,
   a. what were they? _____

   _____
   (name and brand)

   b. how often did he/she take them? _____

   _____

   c. how long did this continue? _____

   _____

   d. do you intend to continue them? _____

   _____

4. How often does your child have the following:
   a. lambs fry or other liver dishes _____
      how much? _____

   b. liver paste or pate _____

how much? _____

c. fish oil emulsions, _____
such as cod liver oil
how much? _____

5. Doctor's (GPs) name _____

address _____

_____

6. Please bring questionnaire and consent form with you to the appointment

7. Use Kermode St entrance of ACH. Walk thru to lifts.
Come to Outpatients Department, first floor, Rogerson Bldg.
Turn left as you come out of the lifts and you will see it.

See you there.

EXCLUSION CRITERIA

1. Vitamin A supplementation >RDA, >3 times weekly over past 3 months.
2. Liver >1 per month
3. Contraindicative medical conditions, such as:
congenital heart disease
congenital lung disease
cystic fibrosis
congenital gastrointestinal disorders eg coeliac disease
immunodeficiency

Appendix 6./

THE ADELAIDE CHILDREN'S HOSPITAL INC

NORTH ADELAIDE
SOUTH AUSTRALIA 5006
Telephone: 267 4999
Telex: ACHOSP: 89178
Ext. 553

DR A J. MARTIN, M.R.C.P. (U.K.)
Paediatric Pulmonologist.

AJM:VW

11th October, 1984

Dear Doctor,

Your patient .....has been selected for inclusion into a double blind trial of vitamin A supplementation versus placebo. our records indicate that he/she was admitted to the Adelaide Childrens Hospital with respiratory syncytial virus infection in infancy and information from several studies indicates that this group of children frequently experience minor recurrent wheezing subsequently. A previous study conducted by the Department of Community Medicine suggest that vitamin A supplementation may decrease the number of respiratory episodes in a similar group of children.

The purpose of this study is to examine the effect of specific vitamin A supplementation over a twelve month period on this group of children. The dose of vitamin A we propose to use is 600 micrograms which is equivalent to the recommended daily allowance for this age group. We should like to emphasize that this is a double blind study and that the children and parents will not know which vitamin we are using, only that it is a vitamin supplementation programme.

No therapeutic advice or management will be offered to your patient and should he/she require further medical help the initiation of this will not be undertaken by this investigation team. However, if any specific abnormalities are found this information will be communicated to you only, for you to determine further action.

Yours sincerely,

A. James Martin.

DIET INTERVIEW

UR No [ ][ ][ ][ ][ ] visit
1 4 5

batch no [ ][ ]
7

. NAME: _____ [ ][ ][ ][ ][ ][ ] 13

. Date _____ [ ][ ][ ][ ][ ][ ] 19

. Date of birth _____ [ ][ ][ ] 22

. Age _____ [ ][ ][ ][ ] 26

. Height _____ [ ][ ][ ][ ] 30

. Weight _____ [ ] 31

. Vitamin supplement yes/no

type/brand _____ [ ] 32

vitamin A dose _____

frequency taken _____

8. Well during preceding 2 weeks? Yes/no

If no, specify:

NAME

24 HOUR RECALL

FOOD	AMOUNT

Name

UR No.

FREQUENCY QUESTIONNAIRE

Code No.	Food	Amount Eaten	Frequency Eaten				COMMENTS: S = Summer W = Winter M = In season O = Out of season
			per day	per week	per month	rarely if ever	
1	Nutrigrain	½ cup					
2	Vital	½ cup					
3	Milk	small glass					
4	Butter	1 tspn					
5	Margarine	1 tspn					
6	Cheese	1 slice (4 x 1)					
7	Yoghurt	½ cup					
8	Egg	1 egg					
9	Cream	1 tblspn					
10	Ice cream	1 scoop					
11	Custard	½ cup					
12	Rich cake: fruit, choc, steam pudd.	1 slice					
13	Milk pudding	½ cup					
14	Fresh orange	1 medium					
15	Fresh peach	1 medium					
16	Fresh apricot	1 medium					
7 18	Other fresh fruit						Type:
19	Canned fruit	¼ cup					Type:
20	Dried apricots	2-3 pieces					
21	Tomato	1 medium					
22	Carrots, raw	1 medium					
23	Carrots, cooked	¼ cup					
24	Pumpkin	¼ cup					
25	Broccoli	¼ cup					
26	Spinach/silverbeet	¼ cup					
27	Corn on cob	1 small					
28	Corn, canned	¼ cup					
29	Capsicum (Sweet pepper)	2 strips 0.5 cm thick					

Name

3 UR No.

FREQUENCY QUESTIONNAIRE

Code No.	Food	Amount Eaten	Frequency Eaten				COMMENTS: S = Summer W = Winter M = In season O = Out of seas
			per day	per week	per month	rarely if ever	
30							
31							
32	Soup	½ cup					
33	Pizza	1 small slice					
34	Mornay dish	1 cup					
35	Liver/lamb's fry	¼ cup (40g)					
36	Quiche	1 slice					
37	Pure orange juice	small glass					Brand
38	Other fruit juice	small glass					
39							
40							
41	Orange juice drink	small glass					Brand
42	Other f/juice drink	small glass					
43	Blackcurrant juice	small glass					
44	Fruit juice cordial	small glass					
45	Aktavite	small cup					
46	Milo/Ovaltine	small cup					
47	Milk shake	small cup					
48	Thick shake	small cup					

Appendix 6.9

Formulation of Supplement

Retinyl palmitate	525 ug re/ml
Raspberry essence	0.5%
Propylene glycol	10%
Tween 80	2%
Sorbitol	30%
Sucrose	30%

Furmulation of the placebo was the same, omitting retinyl palmitate.

RESPIRATORY SYMPTOM DIARY - HOW TO USE IT

The symptom diary is a very important part of our study. It will tell us how much illness your child has over the next 12 months. This is how we would like you to use it:

1. Mark off each day of the month at the top of the page whether or not your child has symptoms.

2. If your child develops symptoms or is feeling upset, indicate which symptoms he/she has by marking the appropriate box with the following numbers:

   "1" if the symptom is mild
   "2" if the symptom is moderate
   "3" if the symptom is severe

The following describe some of the symptoms. If you have any further questions please ask the nurse.

Sneezing: Fill this in if your child sneezes more than 5 times per day.

Sore Throat: A dry, scratchy or sore throat; or if it is painful to swallow.

Fever: Mark this box if your child feels feverish to you; if it is possible to measure his/her temperature, please enter this in the appropriate box.

Wheezing: This is a whistling or squeaky sound when he/she breathes out.

Upsetting Events: These, of course, are not symptoms but we are interested in studying these as well. If something happens in day to day life that your child finds particularly upsetting, fill this box in even if your child is otherwise completely well (for example, being reprimanded by teacher, parent sick or away, loss of absolutely favourite toy, etc.)

Comments: In this important section, please indicate when you take your child to the doctor, or to the hospital. Also indicate any medicine he/she is currently taking, (e.g. Antibiotics, Ventolin, Panadol, etc.). Let us know of anything else which has happened which may affect his/her health, such as an accident.

NAME [illegible] MONTH November YEAR _____ Example

Cross out each day of month. Fill in symptom boxes if child is unwell: 1 if mild; 2 if moderate; 3 if severe

DAY OF MONTH	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
NOSE: sneezing						1	1	1																							
runny/blocked								1	1	2	2	2	1	1					1	2	2	2	2	2	2	2					
THROAT: hoarse																															
sore/scratchy																	2	3	3	2	1	1									
COUGH: dry																					1	1	1	1	1	1	1	1	1		
chesty/moist																															
EAR: pain/discharge																															
BREATHING: wheezing																															
short of breath																															
rapid breathing																															
FEVER																			39°	39°	39°										
VOMITING																															
DIARRHOEA																															
MUSCLE ACHE																															
AT HOME SICK																			✓	✓	✓										
UPSETTING EVENT		✓																										✓	✓		

COMMENTS: (visits to doctor, hospital, medicines)

↑ Visit to doctor
Amoxillin →

NAME Sadie Smith MONTH November YEAR _____ Example

Cross out each day of month. Fill in symptom boxes if child is unwell: 1 if mild; 2 if moderate; 3 if severe

DAY OF MONTH	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
NOSE: sneezing						1	1	1																							
runny/blocked								1	1	2	2	2	1	1					1	2	2	2	2	2	2	2					
THROAT: hoarse																															
sore/scratchy																	2	3	3	2	1	1									
COUGH: dry																					1	1	1	1	1	1	1	1	1		
chesty/moist																															
EAR: pain/discharge																															
BREATHING: wheezing																															
short of breath																															
rapid breathing																															
FEVER																			39°	39°	39°										
VOMITING																															
DIARRHOEA																															
MUSCLE ACHE																															
AT HOME SICK																			/	/	/										
UPSETTING EVENT		/																										/	/		

COMMENTS:
(visits to doctor, hospital, medicines)

Visit to doctor
Amoxillin →

Appendix 6.11

Respiratory Symptom Classification:

Symptom Categories

NOSE	1. sneezing (more than 5 times per day)
	2. runny or blocked nose
THROAT	1. hoarse
	2. sore, scratchy
COUGH	1. dry
	2. chesty/ moist
EAR	ear pain/discharge
BREATHING	1. wheezy
	2. short of breath
	3. rapid breathing
SYSTEMIC	1. fever
	2. vomiting
	3. diarrhoea
	4. muscle ache
	5. at home sick

Appendix 6.12

Respiratory Symptom Classification;
Definition of an Episode of Probable Acute Respiratory Infection

Definition of an episode of illness: one or more days of symptoms, preceded and succeeded by 2 or more symptom free days.

Definition of an episode of probable respiratory infection:

an illness episode comprising one of the following:

1. 3-4 days of continuous symptoms: one or more of nose, throat and cough symptoms
2. 5 or more days of continuous symptoms: any 2 of nose, throat and cough symptoms.
3. 5 or more days of continuous symptoms: one of which was throat category.
4. fever plus any one of nose, throat, cough, ear or breathing categories

Appendix 6.13

SYMPTOM COMPLEX

DURATION	NASAL ONLY	COUGH ONLY	THROAT ONLY	Any 2 of Nasal, thrt. cough, syst.	Any 3 of Nasal, thrt. cough, syst.
1 – 2 DAYS continuous symptoms	DOU	DOU	DOU	UNC	UNC
2+ DAYS intermittant symptoms	DOU	DOU	DOU	UNC	PROB
3 – 4 DAYS continuous symptoms	DOU	DOU	UNC	UNC	PROB
5+ DAYS continuous symptoms	UNC	UNC	PROB	PROB	PROB

Appendix 6.14

NAME	DAYS												EPISODES										ID NUM
COM. DATE _____	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11
Calendar Month																							
Nose : Sneezing																							
Runny/blocked																							
Throat : Hoarse																							
Sore/Scratchy																							
Cough : Dry																							
Chesty/Moist																							
Ear : pain/discharge																							
Breathing : wheezy																							
Short of brth																							
Rapid																							
Fever																							
Vomiting																							
Diarrhoea																							
Muscle Ache																							
At Home Sick																							
Upsetting Event																							

MONTH

IDNUM

EVENT	1	2	3	4	5	6	7	8	9	10	11	12	
Calendar Month													
Probable Infection													
Unclear Infection													
Doubtful Infection													
Prese. Med: AB													
Asthma													
Other													
Dr / OP Visit													
Hosp admit.													
Quality of Diary													

Code: 1 excellent
2 some problems
3 inadequate

Appendix 6.15

NAME _____ UR NO. _____

Batch No. _____

DOSE: 8ml (1½ tspns) supplement ONCE A WEEK. Tick after each dose.

MONTH					
Week 1					
Week 2					
Week 3					
Week 4					

Appendix 6.16

NUTRITION AND RESPIRATORY STUDY

DIET DIARY

In this section of the study, we ask you to keep a record of everything your child eats and drinks over a THREE DAY PERIOD.

First record the food itself and its description (e.g. cordial, lemon; bread, wholemeal) then the amount which is actually eaten. Use teaspoons, cups and tablespoons as measures, as shown in the example on the next sheet.

We ask you to record foods eaten on three consecutive days, beginning on a Sunday, as soon as is convenient. Please return the forms to us as soon as you have finished using the self-addressed envelope.

Remember to write down everything your child eats including sweets, soft drinks and if possible - foods he helps himself to! Extra sheets are included in case you run out of space. You may find this time consuming and we thank you for your continuing patience and cooperation with the study.

NAME Jennifer James

Food Record for DAY 1 Day of week Sunday

OFFICE USE ONLY

Day of Study [ ][ ] ID NUM [ ][ ][ ][ ][ ][ ][ ][ ][ ][ ]

IG	Food Item & Description (brand names is possible)	COOKING METHOD	AMOUNT ACTUALLY EATEN	Food Code	Gms eaten
u	Orange juice drink : Mr Juicy	–	½ cup.		
	Weet bix	–	1 bix		
	Milk on Weetbix	–	½ cup		
	Sugar	–	2 tspns		
m	Twistres		1 packet		
	apple		one quarter		
	chocolate frog	–	1		
on	Cheese sandwich : Bread, wholemeal		2 slices		
	Margarine, Flora		2 tablespns.		
	Cheese, Kraft sliced	–	1 slice		
	Fruit cake (shop-bought)	–	5cm x 3cm piece		
n	carrot fresh	Raw	10cm stick		
	peanuts, salted	–	½ cup		
	orange juice, pure, Berri	–	1 cup		
n	lamb, leg of	Roast	1 small slice		
	peas, frozen	boiled	¼ cup		
	carrots, fresh	boiled	1 tablespn		
	potatoes	roast	1 small		
	peaches, tinned	–	½ cup		
	icecream, Streets vanilla	–	1 scoop		
	milk, low fat	–	1 cup		
	Milo	–	2 teaspns.		

NAME _____

Food Record for DAY

Day of week _____

OFFICE USE ONLY

Day of Study [6][7] ID NUM [8] [1][5] [9][12]

TIME OF EATING	Food Item & Description (brand names is possible)	COOKING METHOD	AMOUNT ACTUALLY EATEN	Food Code [13][16]	Gms eaten [17][20]
					28
					36
					44
					52
					60
					68
					76
				Next Card [13][16]	[17][20]
					28
					36
					44
					52
					60
					68
					76
				Next Card [13][16]	[17][20]
					28
					36
					44
					52
					60
					68

Reasons for Allocation of Scores 2 and 3 in Diary Quality

1. Package returned too clean - bag and diary in perfect condition, obviously unopened.
2. Diary dates at top of page not crossed off, no symptoms ticked.
3. Diary dates crossed off in identical fashion- implies all checked on single occasion.
4. Obvious disinterest from parent, corresponding with no sypmtoms ticked
5. Diaries not returned for variety of reasons

Appendix 7.0

Papers Published by the Candidate

Pinnock, C. B., Douglas, R. M. & Badcock, N. R. (1986). Vitamin A status in children who are prone to respiratory tract infections. *Journal of Paediatrics and Child Health*, *22*(2), 95-99.

Journal of Chromatography, 382 (1986) 290—296
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3284

Note

Liquid chromatographic determination of retinol and α-tocopherol in human buccal mucosal cells

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and

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(First received March 5th, 1986; revised manuscript received June 6th, 1986)

Assessment of vitamin A status is currently focussed on circulating levels of retinol and its binding protein and establishing their relationship to liver reserves of vitamin A [1—3]. The relationship between plasma vitamin A, an insensitive measure of vitamin A status [2], and the functional availability of the vitamin at tissue level has yet to be determined. Measurement of vitamin A in human cheek epithelial (buccal mucosal) cells as a tissue source offers a convenient opportunity to study these relationships in a large number of subjects, including children, because of the non-invasive method of collection of these cells and because buccal mucosa, like other mucosal epithelial surfaces, is sensitive to vitamin A [4—6]. Vitamin E is known to influence the biological availability of vitamin A [7—9], thus a method of assessment of the costatus of these two vitamins at the tissue level is highly desirable.

We describe for the first time a high-performance liquid chromatographic (HPLC) procedure for the detection and quantification of vitamin A (as retinol) and vitamin E (as α-tocopherol) in human buccal mucosal cells. The analysis exploits the cheek cell collection method of McMurchie et al. [10], the extraction procedure of Bligh-Dyer [11], normal- and reversed-phase chromatography and the native fluorescence of vitamins A and E.

0378-4347/86/$03.50

EXPERIMENTAL

Reagents and glassware

All solvents including water were HPLC grade while all other reagents were analytical grade (Ajax Chemicals, Sydney, Australia). Chloroform was further purified by distilling from an all-glass apparatus, rejecting the first 10% and the last 25% of the distillate. All-*trans*-retinol of crystalline purity and *d*-α-tocopherol were obtained from Sigma (St. Louis, MO, U.S.A.).

The stock retinol solution, 1.0 mmol/l (286 mg/l) in absolute alcohol, was stable for at least three months at −20°C. The working retinol solution, 100 nmol/l in absolute alcohol, was prepared fresh on the day of analysis. The stock α-tocopherol solution, 1.0 mmol/l (431 mg/l) in absolute alcohol, was stable for at least three months at −20°C. The working α-tocopherol solution, 1.0 μmol/l in absolute alcohol, was prepared fresh on the day of analysis. The concentration of each working standard was confirmed spectrophotometrically by using their respective absorptivities ($A^{1\%}_{1cm}$) in absolute alcohol: retinol 1780 at 325 nm and α-tocopherol 75.8 at 292 nm.

The internal standard for retinol, 1-naphthol, was obtained from Ajax Chemicals. The stock 1-naphthol solution, 1.0 mmol/l (144 mg/l) in absolute alcohol, was stable for at least six months at −20°C. The working 1-naphthol solution, 100 nmol/l in methanol, was prepared freshly on each day of analysis. The internal standard for α-tocopherol, *d*-tocol, was a gift from Eisai Research Labs. (Tokyo, Japan). The stock tocol solution, 1.0 mmol/l (389 mg/l) in absolute alcohol, was stable for at least six months at −20°C. The working tocol solution, 25.0 nmol/l in methanol, was prepared freshly on each day of analysis.

Sample preparation

Human cheek cells were collected as follows. Subjects were first asked to rinse their mouths with distilled water. Subsequently, cells were collected by vigorously swirling 5 ml of distilled water around the mouth and spitting out the water and contents into a darkened plastic screw-cap jar containing 25 μg butylated hydroxytoluene (BHT). Collecting cycles were repeated three more times until approximately 20 ml had been collected. The resultant cheek cell suspension was filtered through a sintered-glass funnel (Pyrex Size 0, 161—250 μm).

The filtrate was successively aliquoted into a 10-ml glass quick-fit centrifuge tube, centrifuged at 2000 *g* for 10 min and decanted. The cells were resuspended twice in isotonic saline and the washed pellet, remaining following successive centrifuging and decanting, was kept either at room temperature (for up to 2 h) before extraction or stored frozen at −20°C until assay (usually within two weeks).

On the day of the analysis, the thawed cheek cell pellet was reconstituted with 2 ml distilled water by successively vortexing and sonicating for 30 s. Aliquots, 1.2 ml, 0.4 ml and 2 × 0.1 ml, were pipetted into labelled 10-ml glass quick-fit centrifuge tubes for retinol, α-tocopherol and protein determination, respectively.

Extraction procedures

Vitamin A. Known amounts of vitamin A covering the range 0—50 pmol/mg of protein were added in 10 pmol/mg of protein increments to prepared 1.2-ml cell aliquots from a cheek cell pool. To these standard tubes and to each patient sample to be assayed (tube containing a cell pellet aliquot), 3 ml of 1-naphthol working internal standard solution and 1.5 ml of chloroform were added. Tubes were stoppered, sonicated for 10 s, vortex-mixed for 60 s and allowed to stand for 5 min. Water (1.5 ml) and chloroform (1.5 ml) were added. Following gentle mixing by inversion, the tubes were centrifuged at 2000 *g* for 5 min. The infranatant was transferred to another 10-ml quick-fit centrifuge tube and evaporated to dryness at 40°C under a stream of nitrogen.

Vitamin E. Known amounts of vitamin E covering the range 0—500 pmol/mg of protein were added in 100 pmol/mg of protein increments to prepared 0.4-ml cell aliquots from a cheek cell pool. To these standard tubes and to each patient tube containing a cell pellet aliquot, 0.4 ml of a 50 mmol/l solution of pyrogallol in boiled water, 2 ml of tocol working internal standard and 1 ml of chloroform were added. Tubes were stoppered, sonicated for 10 s, vortex-mixed for 60 s and allowed to stand for 5 min. Water (1 ml) and chloroform (1 ml) were added. At this stage the procedure was followed as for cheek cell vitamin A.

Protein in cheek cells

Protein was determined in duplicate on the two 0.1-ml cell aliquots, using the method of Lowry et al. [12].

High-performance liquid chromatography

Liquid chromatographic analyses were performed using a Model 320 liquid chromatograph (Beckman Instruments) equipped with a Model F1000 fluorescence spectrophotometer (Hitachi). The analytical column for retinol was a pre-packed 250 × 4.6 mm I.D. Ultrasphere-Si column, average particle size 5 μm, and the guard column was 50 × 4.6 mm I.D., dry-packed with Spherisorb-Si, 10 μm. Tocopherol was separated on a 250 × 4.6 mm I.D. Ultrasphere ODS, average particle size 5 μm, and the guard column was 50 × 4.6 mm I.D., dry-packed with Ultrasphere ODS, 20 μm (analytical columns and packings, Beckman Instruments).

Vitamins A and E were eluted isocratically with heptane—dioxane (92:8) and methanol, respectively, at a constant flow-rate of 2 ml/min. With detector sensitivity 20, excitation 326 nm, emission 470 nm for retinol and 1, 295 nm and 330 nm, respectively, for α-tocopherol, peak heights were recorded with a 10-mV recorder at a chart speed of 0.5 cm/min.

Residues were dissolved in 300 μl of their respective mobile phases and 250-μl aliquots injected into the chromatograph. Under the above conditions, the retention times for retinol and α-tocopherol were 7.9 and 7.5 min, respectively. A calibration curve was made by plotting peak-height ratios (retinol/internal standard and α-tocopherol/internal standard) against vitamin A and E concentrations, respectively. The intercepts, which represent the endogenous levels of retinol and α-tocopherol in the cheek cell pool, were subtracted from the peak heights of the vitamin A and E standards. The ratio of corrected peak

heights of vitamin standard to internal standard was then calculated and the value of unknown specimens calculated by direct proportion.

RESULTS AND DISCUSSION

Typical chromatograms of an extract of cheek cells from an adult volunteer are illustrated in Fig. 1. For a number of reasons, it was not practical to use the same HPLC conditions for both vitamins. Unlike the excellent separation afforded α-tocopherol and tocol, retinol, at the higher sensitivity setting required for its quantitation, could not be adequately resolved on a reversed-phase column from the gross peak at the beginning of the chromatogram, even with gradient elution and a variety of solvent phase combinations. In addition,

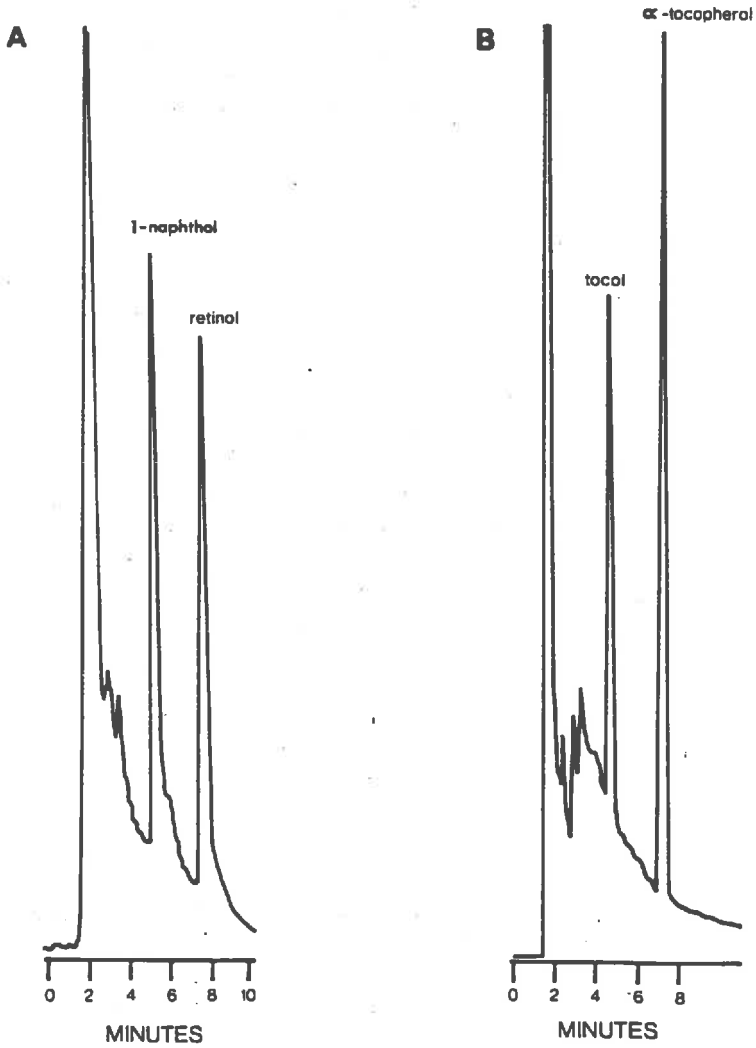

Fig. 1. Liquid chromatograms of extract of human buccal mucosal cells containing 12 pmol retinol per mg protein (A) and 240 pmol α-tocopherol per mg protein (B).

the high fluorescence intensity of α-tocopherol in methanol [13], compared with the hydrocarbon-based mobile phase (which would be necessary for its elution from a silica gel column), and the quite different fluorescent properties of each vitamin necessitated their quantitation using different chromatographic column packings and eluting solvents.

Sensitivity

Detection limit, defined as a signal twice the height of the noise level, was approximately 0.5 pmol retinol per mg protein and 5 pmol α-tocopherol per mg protein. When 1 pmol/mg of protein was added to a cheek cell aliquot with an endogenous retinol concentration of 12 pmol/mg of protein, the peak-height ratio of vitamin to internal standard was increased from 0.92 to 1.00; similarly, when 50 pmol α-tocopherol per mg protein was added to a cheek cell aliquot having an endogenous α-tocopherol concentration of 240 pmol/mg of protein, the peak-height ratio of vitamin to internal standard was increased from 1.69 to 2.04. Concentration and peak height were linearly related throughout the concentration ranges investigated, 1—50 pmol retinol per mg protein and 50—500 pmol α-tocopherol per mg protein. This adequately covers the range of vitamin A and E concentrations in cheek cell samples from normal children and adult controls.

Choice of reference standard

In selection of a reference standard, wet weight, dry weight, cell numbers and protein were considered. Of these, protein was the most reproducible [coefficient of variation (C.V.) 3%] and was correlated with dry weight by a factor 0.811 (n=18). Protein determination also provides increased sensitivity when cell yields are low, a not uncommon occurrence when collections are taken from young children.

Reproducibility

To assess the reproducibility of the method, five replicate samples from a cheek cell pool were analysed on each of five different days. For the five within-day replicates, the mean retinol concentration was 13.7 pmol/mg of protein and the C.V. was 10.6%; the mean α-tocopherol concentration was 381 pmol/mg of protein and the intra-batch C.V. was 9.8%. The corresponding between-day values were: 12.6 pmol retinol per mg protein, C.V. 12.2% and 390 pmol α-tocopherol per mg protein, C.V. 11.1%.

Analytical recovery and stability of preparations

The two vitamins were found to be stable for up to ten weeks in the washed cell pellet when prepared and stored as described above. Freezing the cheek cell collection, as received, was not attempted because of almost certain cell lysis, and storage of the whole cell collection at 4°C produced unpredictable losses of retinol and α-tocopherol, presumably from bacterial action. BHT, at the concentration employed, prevented possible losses of vitamins A and E during cell storage at −20°C. It was preferred to pyrogallol as an antioxidant at the cell collection and storage stage because of its greatly reduced handling toxicity. However, inclusion of pyrogallol during the vitamin E extraction was

essential in preventing excessive and unpredictable losses from oxidative degradation of α-tocopherol. In the absence of pyrogallol, recovery varied between 10 and 80% compared with near complete recovery (94—103%) when pyrogallol was added. Analytical recoveries of retinol were good (> 89%) without pyrogallol and were not improved by its addition, hence we elected to add none in that extraction procedure (Table I).

The high extraction recoveries of vitamins A and E from cheek cells were achieved using the Bligh—Dyer method. We failed to achieve reproducible and quantitative results with hexane procedures previously reported for the extraction of fat-soluble vitamins from plasma [14—17] and somatic afferent receptor cells [18].

TABLE I

RECOVERY OF ADDED RETINOL AND OF ADDED α-TOCOPHEROL TO BUCCAL MUCOSAL CELLS

Compound	Concentration (pmol/mg of protein)			Mean recovery (%)
	Endogenous (mean)	Added	Found (mean)	
Retinol	13.7	10.0	22.0	93
		25.0	34.4	89
		50.0	62.4	98
α-Tocopherol	381	100	452	94
		250	650	103
		500	890	101

Selectivity

The identity of retinol and α-tocopherol in cheek cells was assessed by comparing their retention times to those of the corresponding reference compounds. Sharp and isolated peaks with retention times identical to retinol and α-tocopherol were obtained when these vitamin standards were added to cheek cells, extracted and chromatographed. Moreover, the peak heights were modified as expected from the two original relative concentrations. This co-chromatographic agreement was always found regardless of the chromatographic conditions (different mobile phases and C_{18} columns).

The method employed in this study for the sampling of human cheek cells yielded sufficient material (>25 μg protein per 1-ml cell collection) to allow the analysis of vitamins in cheek cells from children as young as 5 years of age. To lessen direct contamination from food, drugs, vitamin preparations, etc., and because cells are removed from buccal mucosa during eating, collection was made on fasting subjects. By filtering the resultant cheek cell suspension through a sintered-glass funnel, foreign material, such as food particles which may not have been removed by the initial rinse, was separated from the cheek cell filtrate. The cell pellet was washed twice with isotonic saline to exclude possible contamination from saliva.

The method described was developed as part of an on-going epidemiological study in which the relationship between vitamin A status and incidence of respiratory tract infections is being examined [19]. Preliminary investigations suggest that buccal cell retinol may prove a sensitive index of vitamin A status.

The reliability of the method and the relationship to other sensitive measures of vitamin A status will be reported elsewhere. The present investigation has shown liquid chromatography with fluorescence detection to be a highly sensitive and selective method for the analysis of vitamins A and E in buccal mucosal cells.

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Errata

Abstract

Addendum: " It is concluded that oral vitamin A supplementation is unlikely to change either plasma levels of vitamin A or respiratory morbidity in respiratory-prone children who have adequate vitamin A in the diet. However vitamin A status of the second group studied, namely respiratory-prone children who were hospitalized with RSV bronchiolitis in infancy, was low. The study therefore raises questions about the role of non-dietary factors in determining vitamin A status, and the possibility cannot be ruled out that low plasma retinol levels contribute to the high rates of respiratory morbidity observed in this population."

Chapter 1

Page 4: para1, line11, delete 'a,b'
para 2, line 1, insert 'viral' before 'upper respiratory illness'
para 2, line 4, for 'Chandra 1977' read 'Chandra and Newberne, 1977a,b.'

page 5: para 2, lines 5 and 12, for '1977' read '1977a'

page 6: para 1, line 12, after 'normal' insert '(self-perceived as different from "the average")'.

page 8: para 3, line 5, for '1986a,b' read '1986', for 'three' read 'two'.

Chapter 2

Page 14: para3, line 6, for 'Roels et al 1963' read 'Roels et al 1969'

Page 15: para 2, line6, after 'retinol binding protein' insert '(RBP)'.

Page 16: para 1, line 7, for 'Klopp 1985' read 'Klopp 1986'.

Page 30: para 3, line 8, for 'Lancet, 1987'read 'Anonymous, 1987'.

Page 35: para 2, line 3, delete'a,b'.

Chapter 3

Page 43: Para 2, line 6, after 'procedures' insert 'The proposal that buccal cell vitamin A may be useful as an indicator of vitamin A status, problem solving in the analytical development phase, the design of reliability and validation studies and all data analysis were carried out by the author'

Page 51: Table 3.1, insert footnote '** $p<0.01$'

Page 52: line 4, for 'duplicated' read 'duplication', then insert ", U is the common effect, or component of variation shared between all observations".

Page 56: Table 3.4, insert footnote '* $p<0.05$, ** $p<0.01$'

Page 57: Table 3.5, insert footnote '* $p<0.05$'.

Page 64: Para 1, line 15, after 'ALLVEG).' insert ' The experimental design, all detary interviews and data analysis in the Pilot study were carried out by the author.'

Chapter 4

Page 65: Para 3, line 6, after 'relationship' insert '(Table 4.2)'.

Page 67: After para 1, insert " b. Measurement of respiratory symptoms. Use of a retrospective estimate of respiratory morbidity based on parent recall poses a number of problems. Variability in recall ability, in interpretation of respiratory symptoms as an "episode" and in awareness of their child's health status over a long period make the measure problematic at best. Prospective recording on a daily basis with symptom based prompts overcome some of these problems and thus a daily diary method supplemented by interview was used subsequently."
Para 2, line 1, for ' b' read 'c'.

Page 68: para 2, line 17, after'vitamin A' insert 'deficiency'

Page 74: figure 5.1, insert footnote T_0: time of beginning supplementation'
T_1: time of conclusion of supplementation'

Page 94: para 3, line 4, after disorders insert '(including compromised immunocompetence)'.

Page 98: para 3, line2, after 'whooping cough' insert'. These conditions are grouped under the heading "lower respiratory illness" in this study, although it is recognised that a minority of children who experienced croup or whooping cough only would not have had a lower respiratory illness as it is commonly defined'.

Chapter 5

Page 108: para 2, line 12, after 'population' insert ' Passive smoking has been associated with respiratory morbidity during the first 2 years of life, but not subsequently (Fergusson and Horwood, 1985).'

Page 110: para 3, line 12, after 'days' insert '(this is consistent with earlier power calculations in which a population size of 200 was needed to detect a 20% difference in symptom days with 80% power)'

Chapter 6

Page 134: para 1, line 11, for 'primary school' read 'high school'.

Page 126: para 1, line 2, after 'study' insert "using an Ohio 842 Rolling Seal Spirometer attached to a Hewlett Packard Recorder"
para 1, line 6, after ' bronchodilator' insert. "Percent predicted was determined from an algorithm correlating height with lung function measures derived from spirometry of 60 healthy Adelaide children (unpublished observations)"

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Page 190: para 6, line1, for 'Olabukunola I.K.' read 'Kogbes O.I.'

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