EVALUATION OF THE PREVALENCE AND PATHOPHYSIOLOGY OF
SUBCLINICAL VITAMIN A DEFICIENCY IN MALABSORPTIVE STATES OF
CHILDHOOD.

CATHERINE ANNE BOUCHARD

Master of Philosophy

THE UNIVERSITY OF ASTON IN BIRMINGHAM

June 1989

-1-

The University of Aston in Birmingham.

Evaluation of the Prevalence and Pathophysiology of Subclinical Vitamin A Deficiency in Malabsorptive States of Childhood.

Catherine Anne Bouchard   Master of Philosophy   1989

SUMMARY

The prevalence of subclinical vitamin A deficiency was investigated in children with chronic liver disease, cystic fibrosis, and gastrointestinal disease, all of whom received routine vitamin A supplements. Low serum vitamin A levels were found in patients with liver disease, and although a correlation was noted between the severity of liver disease and serum vitamin A levels, age of onset and duration of the liver disease may also influence vitamin A status.

The major factors thought to cause vitamin A deficiency in such patients, malabsorption and retinol-binding protein (RBP) status, were investigated. Even though responses to an oral test dose of vitamin A were similar in a number of patients, the long term response to regular daily oral supplements was found to be varied, possibly as a result of differences in RBP status.

Using successful liver transplant recipients as controls of similar age, serum total RBP levels were found to be reduced in patients with chronic liver disease, but not cystic fibrosis. However, serum total RBP levels in liver disease appeared to remain just within the normal range for age. Although apparently quantitatively normal, the serum RBP may have a reduced affinity for vitamin A, possibly resulting in reduced transport and therefore low serum vitamin A levels.

Subclinical vitamin A deficiency was most prevalent in severe liver disease where oral vitamin A requirements appeared to be highly individual, therefore daily doses of vitamin A should probably be based on a dose per kg of body weight, and may be far in excess of 10,000iu of a water-miscible preparation. Dosage recommendations might be based on severity, duration and age of onset of liver disease, however the relative influences of these factors requires further investigation. Practically, regular measurement of serum vitamin A levels remains the most useful indicator of vitamin A status once the limitations have been recognised; especially when supported by measurement of serum total RBP levels.

KEYWORDS: Vitamin A deficiency, retinol-binding protein, malabsorption, liver disease, cystic fibrosis.

To those children who need, or who have received a liver transplant, whose courage provided the inspiration for this thesis.

My warmest thanks must go to all of the children who attended the Liver Clinic at The Children's Hospital, Birmingham, not only for providing the basis for the thesis, but also for the light relief.

Finally, I am grateful to West Midlands Regional Health Authority and Roche Products Ltd for their financial support.

LIST OF CONTENTS:

-13-

LIST OF FIGURES.

-16-

LIST OF TABLES.

-20-

ABBREVIATIONS.

The following abbreviations have been used throughout the thesis:

A_{450nm}	Absorbance at 450nm
A_{620nm}	Absorbance at 620nm
A_{total}	Total absorbance
A_{pool}	Absorbance of a serum pool
F_C	Calibration factor for serum carotenoids
F_{CC}	Correction factor for serum carotenoids in the determination of serum vitamin A
$F_{Vit\ A}$	Calibration factor for serum vitamin A
$\propto$	alpha
ALT	Alanine transaminase
AP	Alkaline phosphatase
AST	Aspartate transaminase
BSA	Bovine serum albumin
Car	Carotenoids
CMC	Critical micellar concentration
CSF	Cerebro-spinal fluid
DMSO	Dimethybenzadine
EHBA	Extra hepatic biliary atresia
ELISA	Double antibody sandwiched enzyme linked immunosorbant assay
ERG	Electroretinographic examination
Gamma GT	Gamma glutamyltransferase
HPLC	High performance liquid chromatography
LDL	Low density lipoprotein
PBS	Phosphate buffered saline
PT	Prothrombin time
PTT	Partial thromboplastin time
RBP	Retinol-binding protein
apo-RBP	RBP which is not bound to vit A
holo-RBP	RBP which is complexed with vit A
pre-RBP	Precussor of RBP synthesis
CRBP	Cellular RBP
CRABP	Cellular retinoic acid binding protein
RDI	Recommended daily intake
RDR	Relative dose response
RPM	Revolutions per minute
TFA	Trifluoroacetic acid
TMB	Tetramethylbenzadine
TTR	Transthyretin (pre-albumin)
Vit A	Vitamin A
Vit E	Vitamin E
WHO	World Health Organisation

$[\ ]_S$	Serum level
$[\ ]_0$	Serum level at 0 hours
$[\ ]_5$	Serum level at 5 hours
$\bar{x}$	Mean
SEM	Standard error of the mean
V	Intra-assay variation
V_1	Inter-assay variation
r	Regression coefficient
$t_{1/2}$	Elimination half life
*	$p < 0.001$
**	$p < 0.005$
$	$p < 0.05$
^{o}C	Degrees in centigrade
g	Grams
hr	Hours
iu	International units
kg	Kilograms
l	Litres
min	Minutes
ml	Millilitres
mm	Milimetres
nm	Nanometers
n	Number
p	Probability
μg	Microgram
μl	Microlitres
μmol	Micromoles
λmax	Wavelength of maximal absorbance

GLOSSARY OF TERMS

Abetalipoproteinaemia

Abetalipoproteinaemia is a rare genetic disorder in which
the major defect appears to be a lack of lipoprotein B,
resulting in absence of chylomicrons and low density
lipoprotein. The condition is associated with neurological
degeneration and ocular abnormalities, including retinitis
pigmentosa, which may involve deficiencies in vitamin A and
vitamin E.

Alpha$_1$-antitrypsin deficiency

Alpha$_1$-antitrypsin deficiency is a genetic disorder
involving the enzyme alpha$_1$-antitrypsin, the severity of the
deficiency being dependant on the genetic phenotype. The
physiological role of this enzyme remains unclear, but it is
involved in inhibition of the activity of various enzymes
including trypsin, leucocytes, bacterial proteases and
collagenase. Tissue responses to infection and inflammation
are therefore affected by the deficiency of alpha$_1$-
antitrypsin. There is liver involvement in 10-20% of
patients, and this may also involve other genetic factors.
The onset of cirrhosis, if it occurs, is relatively late
compared with EHBA (see below).

Cholestasis associated with parenteral nutrition

Although the exact cause remains unclear, an association between prolonged parenteral feeding and cholestasis has been noted. The cholestasis is usually transient, resolving with the re-introduction of oral feeding, and cirrhosis is rare.

Chronic active hepatitis

A continuing inflammatory lesion of the liver of unknown aetiology, resulting in cirrhosis.

Crigler-Najjar Syndrome

The patients included in the present study suffered from Crigler-Najjar syndrome type II, the less severe form of the syndrome. The inheritance of this genetic disorder is autosomal dominant and the cholestasis frequently occurs during the first year of life although onset may be delayed. Cirrhosis was not present in any of the patients included in the present study.

Extra hepatic biliary atresia (EHBA)

EHBA is a rare disease state in which congenital narrowing or irregularity of the bile duct lumen occurs. The aetiology remains uncertain but may involve a sclerosing inflammatory lesion, however it is unclear whether this develops in the foetus, at birth or immediately following birth. The

condition is characterised most commonly by complete inability to excrete bile. Without surgical treatment (see the definition of the Kasai operation below) rapidly progressive cirrhosis is inevitable within 4-6 months.

Glycogen storage disease

Glycogen storage diseases are inherited metabolic disorders in which the structure and/or concentration of glycogen in body tissues is abnormal, most frequently elevated. The liver may be enlarged due to deposition of excess glycogen but cirrhosis is uncommon except in young adults. Clinical features of the disease are normally associated with hypoglycaemia however malabsorption may occur.

Hyperlipidaemia

Throughout the present thesis hyperlipidaemia refers to congenital elevated serum lipid levels. All patients were asymptomatic, other than the elevated serum lipid levels, and were being treated with a low fat diet.

Idiopathic cirrhosis

Cirrhosis of unknown aetiology which generally follows a period of cholestasis.

Kasai operation (hepatic portoenterostomy)

The Kasai operation is a surgical procedure used in the treatment of EHBA (see above). The operation involves an

anastomosis between the area of the portahepatis and the bowel. Bile duct remnants and fibrous tissue are removed and the hepatic tissue is cut, exposing an area through which bile can drain. A roux-en-y loop is brought up from the jejenum and the end is closed. A side opening is then anastomosed onto the area of cut hepatic tissue.

The Kasai operation is described as successful if bile drainage is achieved, and rapidly progressive cirrhosis does not follow. The success of the operation is highly dependant on the age of the patient, therefore the operation should be completed by the age of 60 days. Following a successful Kasai operation, patients may develop a certain degree of cirrhosis at a later age, however this is not genereally as rapid or extensive as the cirrhosis found in patients following an unsuccessful Kasai operation.

The Kasai operation may be described as unsuccessful if bile drainage is not achieved. This may occur even if the Kasai operation appears to be technically successful, and rapidly progressive cirrhosis is inevitable in such cases.

Malabsorptive states

A patient was said to have a malabsorptive state if the disease was known to be associated with intestinal malabsorption of fat, and therefore fat-soluble vitamins. This included patients with chronic liver disease, cystic fibrosis and gastrointesinal disease. The degree of

-26-

malabsorption was not quantified in the present study.

Neonatal hepatitis

Neonatal hepatitis is a broad term describing cholestasis in infancy of unknown aetiology. The severity and prognosis is very varied, and cirrhosis may or may not develop (see idiopathic cirrhosis). In the present study those patients with unexplained cholestasis but no evidence of cirrhosis were described as having neonatal hepatitis. If cirrhosis was present the patients were included in the group with idiopathic cirrhosis.

Wilson's Disease

Wilson's disease is an autosomal recessive inborn error of metabolism associated with accumulation of toxic amounts of copper in the liver, kidney, brain and cornea. The patient included in the present study was well controlled with little evidence of hepatic involvement.

PART I: INTRODUCTION

CHAPTER 1 : INTRODUCTION TO THE STUDY.

i)   Background to the study.

1.   Individuals   at   risk   of   developing   fat-soluble
      vitamin deficiency.

Two   major   factors   are   thought to be involved   in   the
pathogenesis   of   vitamin   A   deficiency   in   malabsorptive
states.   Firstly,   intestinal malabsorption might reduce the
body content of vitamin A, and secondly, defective synthesis
and/or   release   of retinol-binding protein might result   in
reduced transport of vitamin A.

Absorption of fat-soluble vitamins (A, D, E, and K) may
be   reduced   in any chronic fat-malabsorptive state such   as
chronic liver disease,   cystic fibrosis or   gastrointestinal
disease   (Passmore & Eastwood,   1986).   The   need   for
supplementation   of   these   vitamins   is   well   recognised
(Goodchild, 1986, Weser & Urban 1985, Mowat 1987):-

Vitamin A,   and Vitamin D are routinely administered to
patients   with   malabsorptive   states,   usually   as   a
multivitamin preparation (Goodchild,   1986,   Wesser & Urban,
1985, Mowat, 1987). In general, doses of approximately twice
the   normal requirement have been administered (WHO,   1967),
although higher doses have been recommended in extra-hepatic
biliary atresia (EHBA) (Mowat,   1987, Kaufman et al.,   1987).
However,   the   dosage employed was often based   on   arbitary
criteria,   with   little   consideration   being given   to   the
duration   or severity of the disease state,   or the age   and

-30-

weight of the patient. Furthermore, the effect of the disease state on retinol-binding protein synthesis/release and therefore the ability of such patients to transport vitamin A has frequently been ignored.

Vitamin E has only been administered where a particular problem such as abetalipoproteinaemia (Bieri et al., 1984) or, in some centres cystic fibrosis (Goodchild, 1986) has been recognised. Muller (1986) however, recommended that all patients with malabsorptive states should be supplemented with vitamin E.

Patients with chronic liver disease or gastrointestinal disease, attending The Childrens Hospital, Birmingham received a standard daily dose of 2,500iu of vitamin A (Ketovite liquid). All infants with cystic fibrosis were supplemented with a daily dose of 8,000iu of vitamin A (Abidec drops), whereas multivitamin capsules B.P.C. were administered to older patients with cystic fibrosis, those < 30kg receiving 2,500iu and those > 30kg receiving 5,000iu of vitamin A.

The rationale behind the regimen employed in cystic fibrosis was to administer a higher dose to the younger patients, who were considered to have poor hepatic vitamin A stores in order to promote repletion of these stores. A more palatable capsule was then administered to the older children to maintain the replenished stores.

Relatively low doses of vitamin A have been administered to patients with malabsorptive states attending The Childrens Hospital, Birmingham, therefore vitamin A supplementation might be suboptimal and these patients might remain at risk of developing vitamin A deficiency.

2. Vitamin A supplements.

Water-miscible preparations containing vitamin A such as Ketovite Liquid, Abidec drops or Arovit Liquid are more rapidly and more completely absorbed than oil-miscible preparations such as halibut oil capsules, both in patients with steatorrhoea and healthy controls, since emulsification is not required prior to absorption (Barnes et al., 1950, Kalz & Scafer, 1958). Such water-miscible preparations might therefore be more suitable than oil-miscible liquids, capsules, or tablets for vitamin A supplementation in malabsorptive states.

Pre-formed vitamin A esters, most commonly the palmitate have been used as supplements rather than beta-carotene, since absorption of vitamin A was said to be more complete than that of the precursor, particularly in malabsorptive states (see chapter 2.i.5) (De, 1937, WHO, 1976). This might result from the higher dependence of carotenoids on dietary triglyceride for absorption (WHO, 1967). Moreover, the conversion of beta-carotene into vitamin A in the gastrointestinal mucosal cell is also

-32-

maximised by conjugated bile salts (Goodman & Huang 1965), and bile salt concentration was found to be reduced in many malabsorptive states (Weber et al., 1973, Badley et al., 1970, Hermon-Dowling, 1973). The efficacy of the conversion also declined as the dose of carotenoid increased (Thompson, 1964). In addition, vitamin A but not beta-carotene can be absorbed via the portal route in the absence of bile salts (Forsgeren, 1969). Furthermore the efficiency of vitamin A absorption appears to increase in vitamin A deficiency states (Donoghue et al., 1973). Supplementation with pre-formed vitamin A in a water-miscible preparation was therefore considered in the present study.

Although no carotenoid preparation is commercially available, recent studies investigating the role of vitamin A and carotenoids in the aetiology of cancer have utilised beta-carotene, however these patients demonstrated no malabsorption (Constantino et al., 1988).

3.   Fat-soluble vitamin deficiencies in malabsorptive states.

Deficiencies of vitamins A and E have been described in cystic fibrosis (Fulton et al., 1982), chronic liver disease (Walt, 1984, Russell et al., 1978) and short-bowel syndrome (Howard et al., 1982). Clinical symptoms have not been reported in EHBA despite low serum vitamin A levels (Andrews et al., 1981, Sokol, 1987, Kaufman et al., 1987). However,

specific tests for vitamin A deficiency, such as night
blindness or dark adaptation are limited, particularly in
young children since patient co-operation is essential.
Electroretinographic examination (ERG), which measures
changes in electrical potential in response to light hitting
the retina (Bowman & Rand, 1982) may be completed on
patients above the age of 6 months old (Alvarez et al.,
1983). An abnormal result might be the first sign of vitamin
A deficiency.

The impression cytology technique (Amedee-Manesme et
al., 1988) has demonstrated abnormal conjunctival morphology
in children with chronic cholestasis, who have not received
vitamin A supplementation; abnormalities were associated
with serum levels of 0.03 to 0.52µmol/1. Patients treated
with intramuscular vitamin A (100,000iu as a water-miscible
preparation) every 2 months for at least a year were found
to have higher serum levels (0.66 to 3.00µmol/1) and normal
conjunctival morphology. Abnormal morphology might be the
first sign of subclinical vitamin A deficiency which can be
detected, particularly in young children where other tests
such as an ERG are inappropriate.

Xerophthalmia is not seen in the technically developed
world, however vitamin A deficiency remains a major public
health problem in the third world (WHO, 1976, Sandford-
Smith, 1988, Underwood, 1984). In developed countries night
blindness may be associated with vitamin A deficiency, but

-34-

only in individuals with chronic malabsorptive states (Fulton et al., 1982, Russell et al., 1978).

Subclinical vitamin A deficiency or marginal serum vitamin A levels (Underwood, 1978, WHO, 1982) have been associated with poor growth rate (West et al., 1988), anorexia, poor assimilation of nutrients, reduced resistance to infection (Chandra, 1988, Milton et al., 1987, De Sole et al., 1987) and impaired haematopoiesis (see chapter 2.v.). Although such signs could easily be attributed to a number of factors, particulary where nutrition is poor, animal studies (Bieri et al., 1969) and experimentally induced vitamin A deficiency in adults (Sauberlich et al., 1974, Hume & Krebs, 1949) strongly indicated an association with vitamin A which cannot be ignored (Underwood, 1978).

In view of the known adverse consequences of vitamin A deficiency in patients with malabsorptive states, the present study was designed to investigate vitamin A status in such patients, in particular those presenting with chronic liver disease. Symptoms such as poor weight gain, anorexia and resistance to infection were of particular importance to the patients included in the present study since many were malnourished, requiring an increased calorific intake and growth was poor (Sokol, 1987, Goodchild, 1986, Woolf et al., 1983). Moreover, recurrent infection was common. Considerable emphasis has been placed on nutritional support at The Children's Hospital,

Birmingham, aiming to improve the quality of life of the patients, and in the case of those with chronic liver disease who might require a liver transplant, prepare them for the stress of major surgery. Recurrent infection in the latter would also be expected to reduce the quality of life, and present an additional risk factor, during surgery.

Although measurement of serum vitamin A levels had limitations in terms of specificity and sensitivity in assessing vitamin A status, it remained the most practical biochemical method available (see chapter 11.i) (Underwood, 1984). Serum vitamin A levels were therefore employed to initially assess patients in the present study.

Ethical approval for the present study was obtained from the Central Birmingham Health Authority Research Ethical Committee (Reference Number 1362).

It was hoped that age-matched normal controls would be obtained for the study, since serum vitamin A (Letner, 1984) and retinol-binding protein (RBP) levels (Vahlquist et al., 1975) are affected by age. However ethical approval to obtain blood specimens from healthy children could not be justified or obtained. Several attempts were made to obtain specimens from children undergoing routine, minor surgery, but these were not successful.

iv) Aims and objectives.

A large number of patients with chronic malabsorption
resulting from chronic liver disease, cystic fibrosis or
gastrointestinal disease, might be risk of developing
vitamin A deficiency. Although the need for vitamin A
supplementation was recognised, supplementation had not been
monitored, other than by occasional, random serum vitamin A
level determination. In the present study, a more consistent
approach to serum level monitoring was implemented in order
to assess the incidence of biochemical vitamin A deficiency
amongst such patients.

The effect of oral supplementation with a fixed dose of
vitamin A on serum vitamin A levels was considered.
Furthermore, the response of serum vitamin A levels to
increasing vitamin A dosage was investigated in patients
with chronic liver disease, attempting to achieve normal
serum vitamin A levels and therefore eliminate the risk of
vitamin A deficiency.

The two major factors thought to be involved in the
pathogenesis of vitamin A deficiency in malabsorptive
states, malabsorption and defective transport of vitamin A
by retinol-binding protein (RBP) were investigated. Firstly,
the response to a test dose of vitamin A was studied in a
number of patients. As it became clear that the initial dose
of 5,000iu of vitamin A employed in the absorption tests was
resulting in little response, the dose was increased to

-37-

10,000iu of vitamin A. A dose of 100,000iu of vitamin A was employed in the patient with abetalipoproteinaemia, where malabsorption was known to be particularly severe. Secondly, serum total retinol-binding protein levels were measured in subgroups of the patients to investigate the likelyhood of impaired transport of vitamin A.

Since dosage manipulation was to be considered in chronic liver disease, the relationship between vitamin A and parameters of liver function, particularly serum bilirubin and albumin levels was determined. A correlation between serum vitamin A levels and liver function tests might provide information for the basis of a dosage regimen. Serum bilirubin reflects the degree of cholestasis, and might therefore give some indication of the ability to absorb vitamin A, whilst serum albumin levels indicate the ability of the liver to synthesise proteins. If serum albumin levels were low indicating long term cirrhosis, the synthesis of retinol-binding protein might also be impaired resulting in inaderquate transport of vitamin A. In addition to the standard liver function tests, the relationship with caffeine clearance and the overall severity of disease was determined, since this might allow consideration of the effects of both cholestasis and the ability of the liver to synthesis protein (which may be impaired by cirrhosis) on vitamin A status. of the disease state than single liver function tests.

In summary, the aims of the study were as follows:

i) To establish whether chronic paediatric liver disease, cystic fibrosis, or gastrointestinal disorders were associated with low serum vitamin A levels.

ii) To determine whether routine oral supplementation with fixed daily doses of vitamin A resulted in acceptable serum vitamin A levels.

iii) To determine whether increasing the oral daily dose of vitamin A resulted in elevation of serum vitamin A levels in chronic liver disease.

iv) To consider whether poor serum vitamin A levels, if encountered resulted from malabsorption, and/or defective retinol-binding protein synthesis or release.

v) To establish whether there was a correlation between serum vitamin A levels and disease state in chronic paediatric liver disease.

In addition to the main aims of the present study described above, the following were considered:

i) The possible relationship between vitamin A and Vitamin E.

ii) The possible relationship between vitamin A and zinc.

iii) The feasibility of the impression cytology technique as a routine screening technique for vitamin A deficiency.

iv) Various parameters of vitamin A status were considered as possible alternatives or adjuvents to measurement of serum vitamin A levels in the evaluation of vitamin A status since the limitations associated with measurement of serum vitamin A levels in terms of sensitivity and specificity were accepted (see chapter 11.i.).

The study was conducted in a number of parts as follows:

1. Determination of serum vitamin A levels.

2. Determination of serum carotenoid levels.

3. Absorption of vitamin A.

4. Determination of serum retinol-binding protein levels.

5. Evaluation of the impression cytology technique.

The preliminary findings of parts of the present study were presented at the annual meeting of the British Paediatric Association, in York (April 1988). The abstract from this meeting has been included in appendix i.

CHAPTER 2 :   INTRODUCTION TO THE PHYSIOLOGY AND CHEMISTRY OF

VITAMIN A.

i)   Physiology of Vitamin A.

Vitamin A is an essential nutrient (Bauernfeind, 1980) required for vision (Moore, 1957), growth (Underwood, 1984), maintenance of differentiated epithelia, mucus secretion and reproduction (Goodman, 1984a, Underwood, 1984). It cannot be synthesised within the body but occurs in two forms in the diet (Bauernfeind, 1984). Pre-formed vitamin A, as long chain esters is found in animal tissues (Goodman, 1984a, Olson, 1967), whilst plant tissue is a source of carotenoids, the vitamin A precursors (Moore, 1957) which account for approximately 60-90% of the intake of vitamin A activity (WHO, 1967, Goodman et al., 1966).

1.   Nomenclature.

Naturally occurring compounds with vitamin A activity (other than carotenoids), and synthetic analogues of vitamin A (with or without activity) are termed retinoids (IUPAC-IUB Joint Commission on Biochemical Nomenclature, 1982). Different retinoids can selectively display some, but not necessarily all of the biological activities of vitamin A (retinol). For example, vitamin A acid (retinoic acid) can support normal growth rates and epithelial cell differentiation (Deluca, 1979), however it cannot replace vitamin A as a precursor for visual pigments, or support reproduction (Thompson et al., 1964).

-42-

One international unit (iu) of vitamin A is defined as 0.3µg all-trans retinol. All sources of vitamin A activity can be converted into international units of vitamin A activity (Goodman, 1984, Passmore & Eastwood, 1986, Underwood, 1984):

$$1iu = 0.3µg \text{ all-trans retinol}$$
$$= 0.6µg \text{ beta-carotene}$$
$$= 1.2µg \text{ mixed carotenoids}$$

Throughout this thesis, a distinction has been made between carotene (beta-carotene) and carotenoids. The serum levels of total carotenoids have been measured without distinction between the various types, whereas beta-carotene was used for calibration experiments since this compound was a standard preparation, with well defined vitamin A activity.

2.   Chemistry of vitamin A.

The vitamin A molecule consists of a hydrocarbon chain, with a beta-ionone ring at one end, and an alcohol group at the other (Figure 2.1).
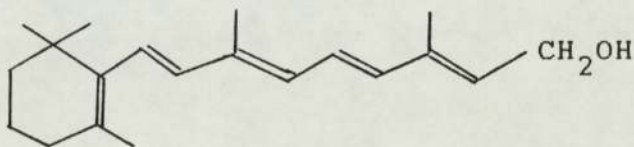
Figure 2.1. represents the structure of the vitamin A molecule.

-43-

The usual form is the all-trans sterioisomer; isomers with a cis-configuration at the C-11 or C-13 position have lower biological activity (Passmore & Eastwood, 1986). The terminal alcohol group can be oxidised in the body to form an aldehyde (vitamin A aldehyde or retinaldehyde) or a carboxylic acid group (Vitamin A acid or retinoic acid).

3.   Chemistry of carotenoids.

Carotenoids are red and yellow fat-soluble pigments. Over 400 have been identified, however most are xanthophylls which have oxygenated substitutions in the ring or side chain, and little or no vitamin A activity (Underwood, 1984). The carotenoid structure must contain a beta-ionone ring with a polyene side chain, containing 11 carbon atoms (Figure 2.1) for vitamin A activity (Underwood, 1984). Bauernfiend (1972) estimated that 50-60 carotenoids could have vitamin A activity, based on their structure. However, few have both vitamin A activity and occur in significant amounts in common foods. The most important carotenoids are beta-carotene, alpha-carotene, gamma-carotene and cryptoxanthin (3-OH-beta-carotene) as shown in Table 2.1.

4.   Conversion of beta-carotene to vitamin A.

Cleavage of the beta-carotene molecule by the enzyme beta-carotene-15-15'-oxygenase in the intestinal mucosal cell (Passmore & Eastwood, 1986) produces 2 molecules of vitamin A, as shown in Figure 2.2 (Olson & Hayaishi, 1965).

beta-carotene

H-C' O
C
H

beta-carotene-15-15'-oxygenase

CH₂OH   CH₂OH
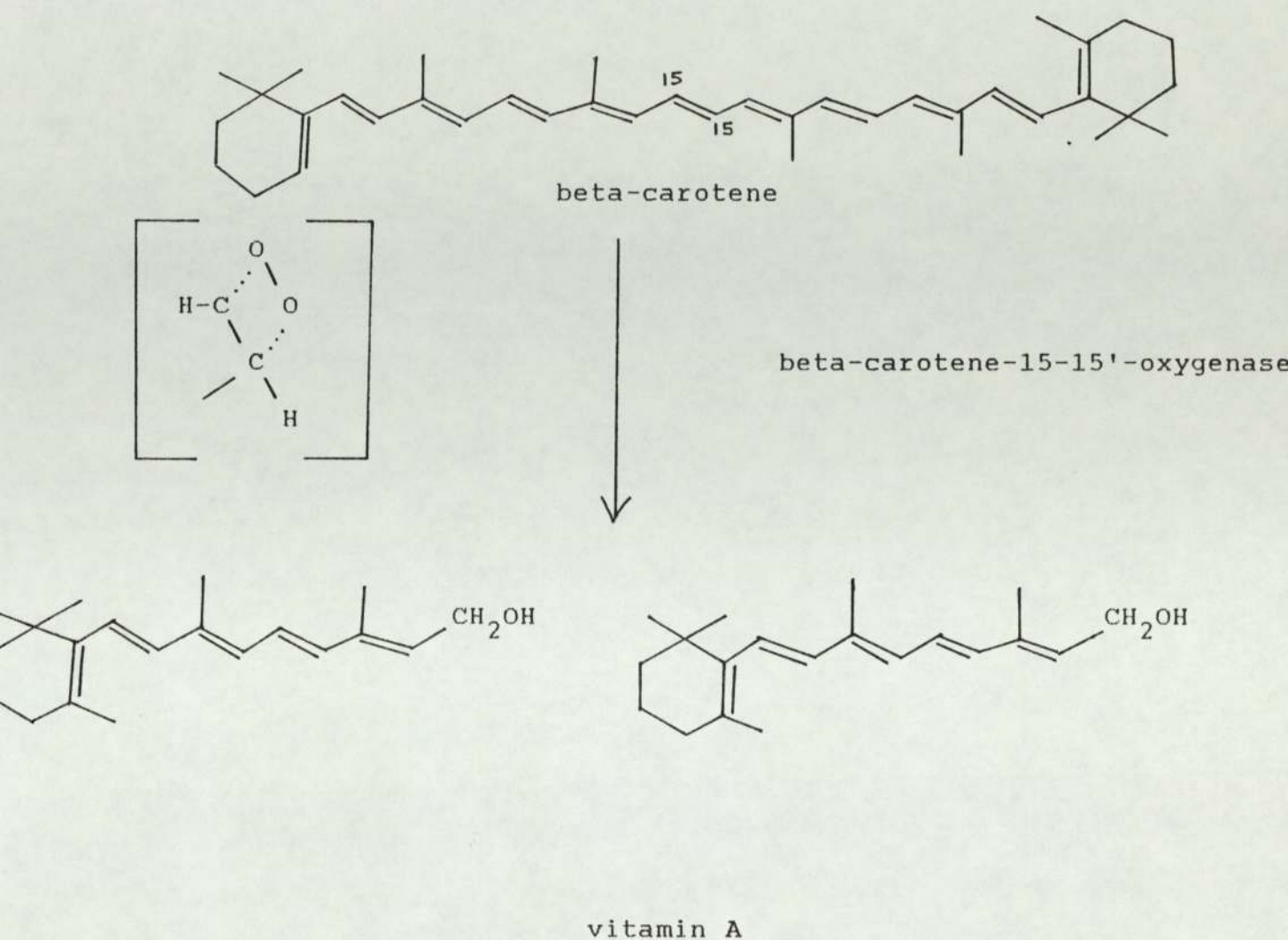
vitamin A

Figure 2.2. represents the cleavage of beta-carotene to produce 2 molecules of vitamin A in the intestinal mucosal cell.

-45-

The conversion may not be complete, therefore prolonged high carotenoid intake results in hypercarotenaemia rather than hypervitaminosis A (McLaren, 1981).

Carotenoid	Vit A activity (%)	Major sources
beta-carotene	100	Red palm oil, carrots, leafy vegetables, tomatoes, fresh apricots bananas, sweet potatoes, apples, pears, orange & juice, pineapple, figs, grapes, watermelon, straw-berries, wheat, corn, pasta products eggs, fish.
alpha-carotene	50-54	Carrots, corn, green peppers, potatoes, apples, peaches, oranges watermelon, cherries, figs, bananas, pineapple, pasta, red palm oil.
gamma-carotene	42-50	Carrots, sweet potatoes, corn, tomatoes, apricots, watermelon.
cryptoxanthin	50-60	Yellow corn, green peppers, lemons, oranges, prunes, apples, apricots, peaches, strawberries, pineapple, pasta, eggs, poultry.

Table 2.1. shows the major dietary sources of carotenoids and their relative vitamin A activity.

5.   Absorption of vitamin A and carotenoids.

A detailed review of the intestinal absorption of vitamin A has been provided by Goodman and Blaner (1984). Absorption of vitamin A and carotenoids occurs via the same route and is dependent on a co-ordinated series of

intestinal, biliary, and pancreatic events as summarised in Figure 2.3 (Goodman & Blaner, 1984).

Although occurring by the same route, the absorption of carotenoids appears to be less consistent than that of vitamin A. The source of the carotenoid for example, results in considerable variation (De, 1937); intestinal absorption of carotenoid from arachis oil was found to be considerably greater (74%) than from sliced carrots (25%). (Hume & Krebs, 1949). Furthermore the type and amount of dietary fat, also affects absorption (Hollander & Rubie, 1978).

a.   Luminal phase of absorption.

Triglyceride represents 90% of dietary fat and must undergo lipolysis within the proximal jejunum to produce monoglyceride and free-fatty acids (I) (Friedman & Nylund, 1980). Following emulsification, dietary vitamin A esters are also hydrolysed to form vitamin A (II) which is solubilised along with carotenoids and the other products of lipolysis, by conjugated bile salts to produce mixed micelles (III). These micelles diffuse to the surface of intestinal epithelium, prior to uptake across the microvillus membrane (IV). Uptake is maximal at the critical micellar concentration (CMC) of bile salts (approximately 3-4mM) (El-Goreb & Underwood, 1973).

Conjugated bile salts also facilitate the action of pancreatic lipase (Friedman & Nylund, 1980) (I), and

-47-
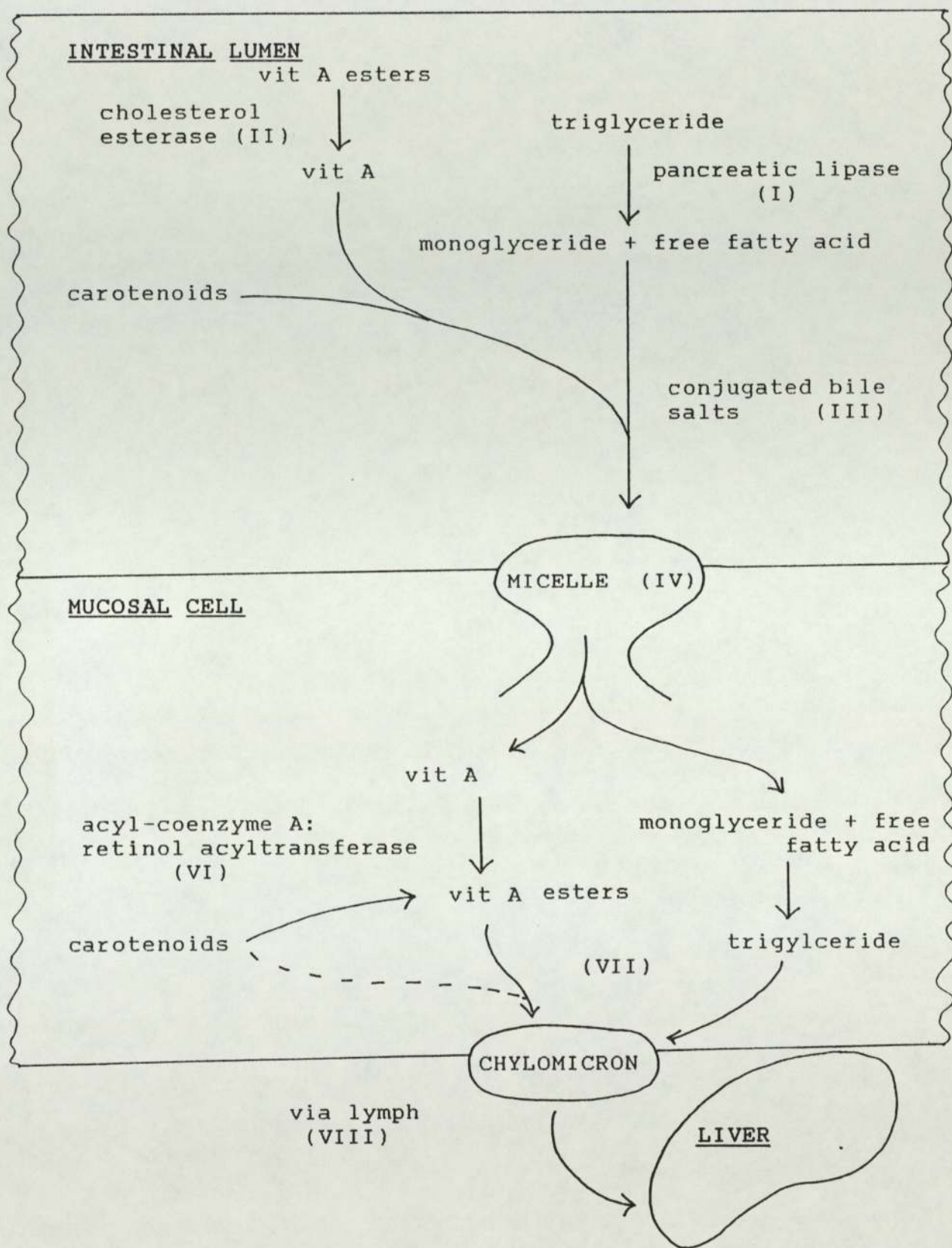
Figure 2.3. represents the stages involved in the absorption
of vitamin A & carotenoids.

maximise the conversion of carotenoid to vitamin A in the intestinal mucosal cell (Goodman & Huang, 1965, Olson, 1964), probably because bile salts are necessary for the interaction of carotenoid with the cell membrane (El Goreb et al., 1975) (See below). Bile salts are also necessary in vitro for the activity of vitamin A palmitate hydrolase, the enzyme responsible for vitamin A mobilisation within the liver (Harrison et al., 1979). Conjugated bile salts therefore play a central role in the absorption of vitamin A and carotenoids.

b. Intracellular phase of absorption.

As previously stated, beta-carotene is primarily converted into vitamin A within the intestinal mucosal cell (V) (Goodman et al., 1966, 1967), although a small proportion of carotenoid is absorbed unchanged (Goodman & Blaner, 1984). The conversion of beta-carotene to vitamin A has also been demonstrated in the liver (Olson & Hayaishi, 1965).

Virtually all of the vitamin A, either newly formed from carotenoids or ingested is re-esterified with long chain fatty acids (VI) (Goodman et al., 1966). The reaction is catalysed by acyl-coenzyme A:retinol acyltransferase enzyme (Helgerud et al., 1983). The vitamin A esters, predominantly the palmitate are incorporated into chylomicrons (VII), along with any unchanged carotenoid,

triglyceride (which is reformed from monoglyceride and fatty acids within the mucosal cell), and apolipoproteins. The chylomicrons are then secreted into the lymph (VIII).

c.   Chylomicron metabolism.

The chylomicrons reach the lymph via the intestinal lacteals (Friedman & Nylund, 1980), and ultimately pass into the serum compartment (mainly through the thoracic duct), where chylomicrons are reduced to chylomicron remnants (Redgrave, 1970). The chylomicron remnant is low in triglyceride and rich in cholesterol esters, phospholipid, and protein. Vitamin A esters are almost completely retained within the chylomicron remnant which is rapidly, and almost entirely removed from the circulation by the liver (Goodman & Blaner, 1984).

The vitamin A esters can undergo exchange with lipoproteins in the serum, however this is probably not physiologically important since the remnant undergoes rapid hepatic uptake (Friedman & Nylund, 1980); Blomhoff and co-workers (1982) demonstrated a half life of 10 minutes for vitamin A esters in serum, 80-90% of the esters reaching the liver, within 30 minutes.

There is little data available about the distribution of carotenoids within the chylomicron (Underwood, 1984). Carotenoids are associated with low density lipoproteins (LDL) within the serum, however the method of transport

-50-

between the chylomicron and the LDL is unknown. There is a correlation between serum concentration of carotenoid and LDL, therefore hypercholesterolaemia is associated with high carotenoid levels (Goodman & Blaner, 1984).

d.   Hepatic uptake and storage.

Hepatic uptake of vitamin A is thought to occur by active endocytosis. This is followed by lysomal degradation of the chylomicron remnant constituents. Hydrolysis of vitamin A esters by vitamin A hydrolase is followed by re-esterification (Goodman, 1965). The hydrolysis and re-esterification both require the presence of bile salts in vitro, presumably since bile salts are necessary for solubilisation of an insoluble substrate (Harrison et al., 1979). At least 95% of hepatic vitamin A occurs in esterified form, predominantly the palmitate (Futterman & Andrews, 1964). Limited information is available about the enzymatic processes involved in the esterification, although an acyl coenzyme A:vitamin A acyl transferase may be involved (Helgerud et al., 1983).

Hepatic storage of vitamin A as esters, is extensive; up to 90% of total body vitamin A is contained in the liver (Raica et al., 1972). The esters of vitamin A are initially stored in the hepatocytes (parenchymal cells) (Blomhoff et al., 1982), however some of the esters then pass to fat-storing cells (non-parenchymal cells) (Olson & Gunning,

-51-

1983). In hypervitaminosis A (excess vitamin A intake), storage within the fat-storing cells increases (Mclaren, 1981). Conversely, in vitamin A deficiency vitamin A appears to be immediately mobilised from the hepatocyte. The effect of vitamin A status on storage and mobilisation of vitamin A has been summarised in **Figure 2.4.**

Within the liver the vitamin A esters are bound to cellular (cytosol) retinol-binding protein (CRBP), a specific intracellular protein which differs from serum retinol-binding protein (RBP) (Chytil & Ong, 1979). CRBP also demonstrates vitamin A ester hydrolase activity, and Chytil & Ong (1979) suggested that it probably facilitates movement of vitamin A from the site of storage to the site of interaction with RBP, prior to transport to the site of action.

Hydrolysis of vitamin A esters must occur prior to mobilisation, since serum RBP has no affinity for the esters (Cogan et al., 1976). The process of hydrolysis, and RBP synthesis and secretion appear to be co-ordinated (Goodman 1984b).

Storage of carotenoids is not limited to the liver, but is also deposited in the adrenal glands, adipose tissue and in the male, the testes (Racia et al., 1972). The exact role of the carotenoids in the body remains unclear, although low serum levels of carotenoids have been associated with the

-52-

a.   Normal vitamin A status.

vit A esters   Serum

vit A esters

          ⟶ vit A ──── RBP ────⟶ holo-RBP ─────⟶

stored vit A esters   TTR ──────────────⟶

b.   Hypovitaminosis A.

vit A esters

vit A esters
                          RBP
          ⟶ vit A ────⟶ holo-RBP ─────⟶
stored vit A esters   TTR ──────────────⟶

c.   Hypervitaminosis A.

vit A esters

vit A esters ══════════════════════════⟶
                          RBP
          vit A ──────⟶ holo-RBP ────⟶
                          TTR ──────────⟶
stored vit A esters
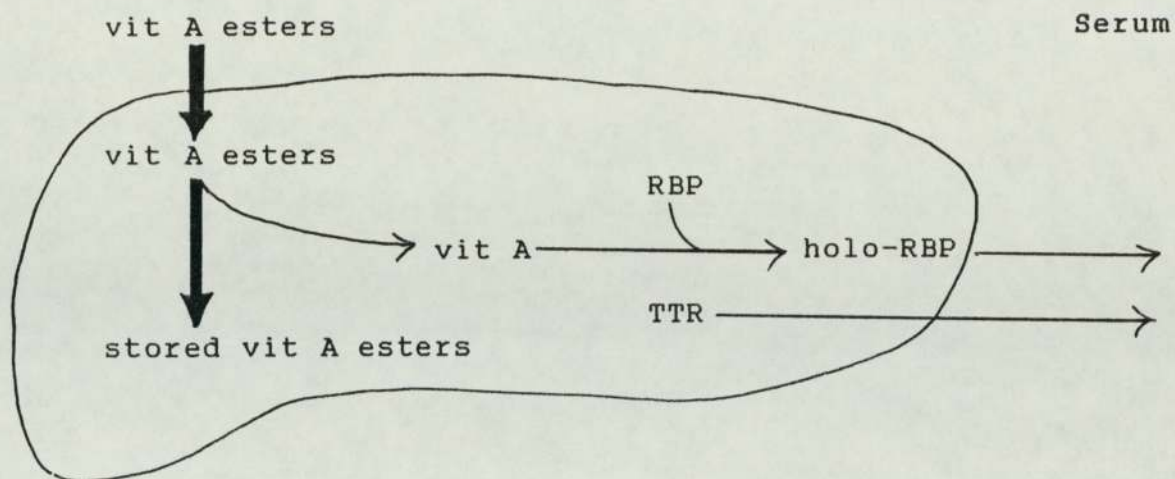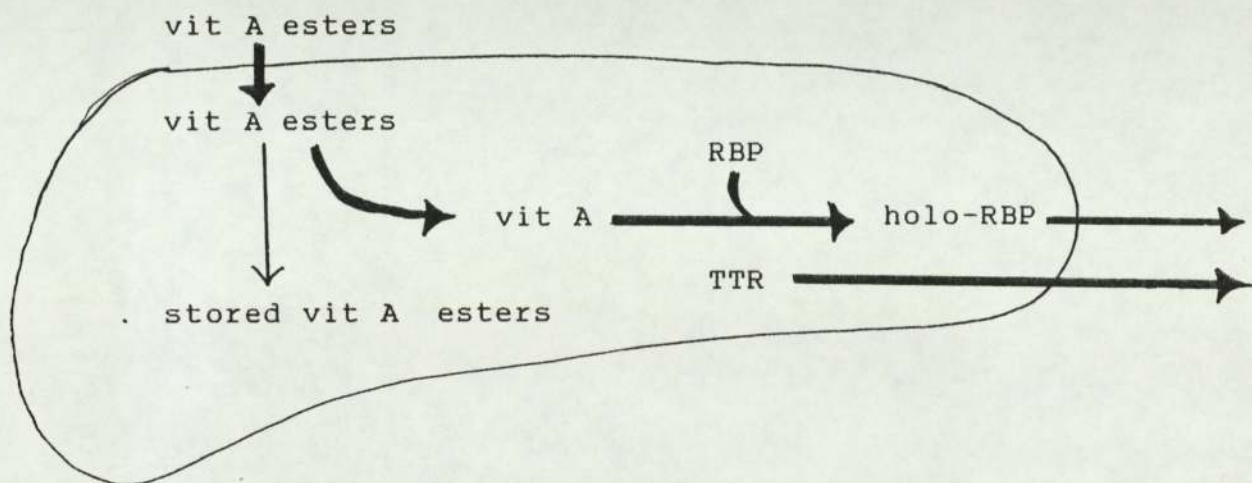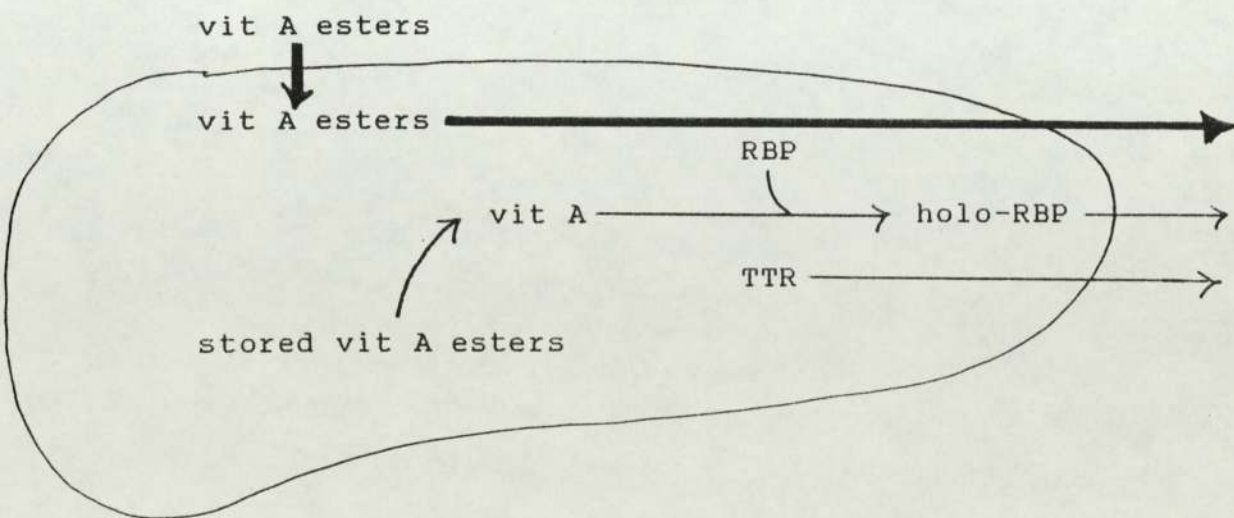
Figure 2.4. represents hepatic uptake and release of
ingested vitamin A under conditions of normal (a.)
depleted (b.) and saturated (c.) hepatic stores of
vitamin A.

development of various cancerous states (Constantino et al., 1988, Meguild et al., 1988). The carotenoid deposits may be converted into vitamin A (see section i.4. above), either in the liver or at the sites of storage (Olson & Hayaishi, 1965).

e.   Transport of vitamin A within serum.

Vitamin A (as the alcohol) is transported within post-absorptive serum, bound to a specific transport protein, RBP (Kanai et al., 1968). RBP consists of a single polypeptide chain (180-186 amino-acid sequence), with a molecular weight of 21,000, and a single binding site for one molecule of vitamin A. Most of the RBP in serum circulates as a complex with vitamin A (holo-RBP), which also interacts with transthyretin (TTR, pre-albumin) in a 1:1 complex (Goodman, 1984b). Vitamin A is not essential for this interaction, although affinity of TTR for apo-RBP (RBP not bound to vitamin A) may be lower in the absence of vitamin A. In addition to vitamin A, the RBP-TTR complex also acts as a transport protein for thyroid hormones. The interaction of holo-RBP and TTR appears to stabilise the interaction with vitamin A (Peterson, 1971). The synthesis and metabolism of RBP is discussed in section ii below.

f.   Metabolism and excretion of vitamin A.

Catabolism of vitamin A would appear to be irreversible, resulting in the production of increasingly

-54-

polar metabolites, which are mainly excreted in the urine (WHO, 1976). However, it is not clear whether the catabolism is uniform for all tissues (Underwood, 1984).

ii) Physiology of retinol-binding protein.

1. Synthesis of retinol-binding protein.

Retinol-binding protein (RBP) is synthesised and secreted as holo-RBP (bound to vitamin A) by the liver (Smith et al., 1978). The holo-RBP complex circulates in serum bound to transthyretin (pre-albumin), which is secreted by the liver at a separate location (Figure 2.5). Parenchymal liver disease can cause reduced synthesis of both RBP and transthyretin, depending on the extent of tissue damage (Smith & Goodman, 1971).

Synthesis of RBP requires the co-ordination of the bio-synthesis of RBP from pre-RBP, processing of the newly formed RBP, translocation to the site of interaction with vitamin A, and formation of holo-RBP, followed by secretion of the holo-RBP. These processes appear to be controlled by three major factors:

a. Vitamin A status

Vitamin A deficiency inhibits the secretion of holo-RBP resulting in reduced serum levels of total RBP. Accumulation of apo-RBP within the hepatocyte indicates that the synthesis of RBP is unaffected by vitamin A

-55-
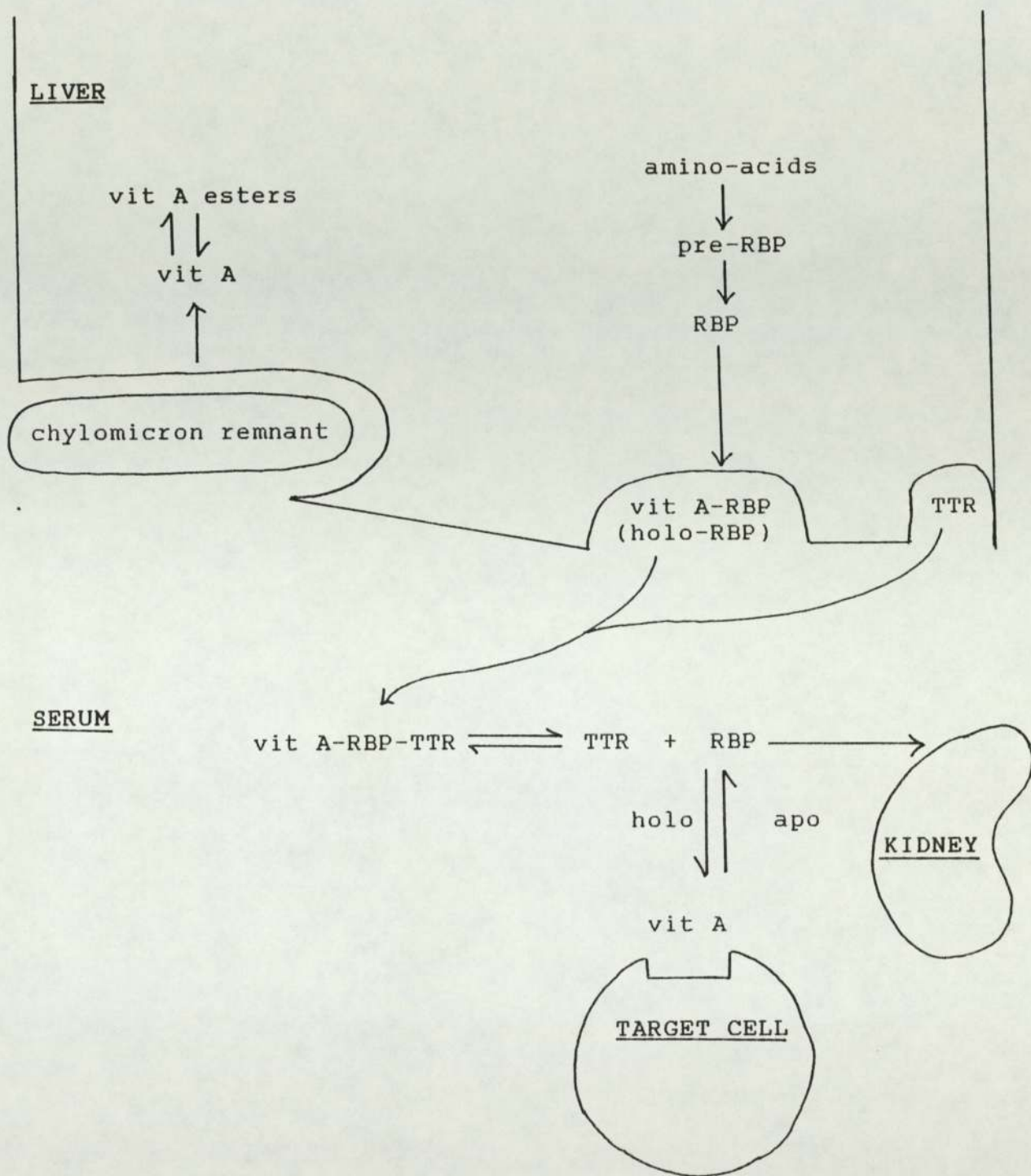
Figure 2.5. represents hepatic synthesis and release of RBP, and it's role as the transport protein for vitamin A.

status (Muto et al., 1972, Smith et al., 1973a). In contrast, vitamin A repletion stimulates rapid secretion of the apo-RBP, the response being proportional to the dose of vitamin A administered. Since the increase in serum total RBP is accompanied by a fall in hepatic apo-RBP (Smith et al., 1973a), repletion appears to cause release of existing RBP, rather than de novo synthesis (Smith et al., 1975) (see Figure 2.4 above).

b. Glucocorticoids

Cortisol, corticosterone and the synthetic analogue, dexamethasone stimulate RBP synthesis in hepatoma cells in culture, however the precise role of glucocorticoids in vivo remains unclear (Borek et al., 1981).

c. Protein intake

Protein energy malnutrition may result in reduced synthesis of export proteins including RBP due to a lack of amino-acid substrate and energy (Smith et al., 1973a, Arroyave et al., 1961, Ingenbleek et al., 1975).

In contrast, transthyretin synthesis is not influenced by vitamin A status (Navab et al., 1977, Smith et al., 1978) or glucocorticoids (Boreck et al., 1981).

In rats, the rate of RBP synthesis was found to be unaffected by vitamin A status (Soprano et al., 1982). These findings indicate that degradation of RBP within the liver must be increased to maintain the elevated steady state of hepatic RBP. However the mechanism of hepatic RBP catabolism is unknown.

2.   The interaction between apo-RBP and vitamin A.

The amount of vitamin A available for complexing with apo-RBP is dependent on several factors:

a.   Vitamin A intake since this influences the amount of vitamin A available in the liver.

b.   The manner in which vitamin A is transported by cellular retinol-binding protein (CRBP) from the hepatic site of storage to that of hydrolysis, and then to the site of interaction with apo-RBP.

c.   The manner in which the vitamin A molecule is presented to the membrane bound apo-RBP.

3. Delivery of vitamin A.

a.   To sites of action.

Smith, and co-workers (1975) demonstrated that the concentration of RBP in rat tissue, other than liver, kidney, and serum was very low, and probably resulted from contamination with residual serum. Surface receptors for RBP

are thought to occur in tissues in which vitamin A is active (Smith & Goodman, 1971). The affinity of the receptor for RBP is reduced once the vitamin A molecule is discharged, resulting in removal of the apo-RBP from the receptor, following delivery of vitamin A. The apo-RBP is then rapidly catabolised (see below).

b.   Foetal delivery of vitamin A.

The amount of vitamin A delivered to the foetus was found to be consistent despite wide variations in maternal intake, indicating a regulatory function of RBP (Takahashi et al., 1975). Furthermore, changes in foetal cellular retinol-binding protein (CRBP) and the corresponding protein for retinoic acid (CRABP) indicate that requirements for vitamin A change during foetal development (Chytil & Ong, 1984, Takahashi et al., 1975).

4.   Catabolism of retinol-binding protein.

The kidney plays an important role in RBP catabolism (Smith & Goodman, 1971). The apo-RBP released following vitamin A delivery is not bound to transthyretin and is rapidly excreted by glomerular filtration. The biological half life of unbound RBP is approximately 4 hrs, therefore only a small amount of unbound apo-RBP (approximately 4% of total RBP) appears in serum.

5.   Physiological roles of retinol-binding protein.

The physiological roles of RBP can be summarised as:

a.   Regulation of vitamin A mobilisation from hepatic stores.

b.   The transport of vitamin A, in serum, to the peripheral sites of action.

c.   Protection of the vitamin A molecule from oxidation in serum.

d.   Delivery of vitamin A to specific sites on target cells, facilitating full utilisation of vitamin A, and possibly preventing toxic side effects (Vahlquist et al 1982).

e.   Delivery of vitamin A from the mother to the foetus.

iii) Pathophysiology of vitamin A deficiency.

Dietary insufficiency of vitamin A only occurs in under-developed countries (Underwood, 1984). Vitamin A deficiency may however also occur in technically developed countries in association with disease states in which fat-malabsorption occurs, or in which RBP status is affected. It is also possible, although not proven, that certain disease states may alter total body requirements, utilisation at a cellular level, or excretion of vitamin A (Mezey, 1978).

The 2 major factors which may contribute to the development
of vitamin A deficiency are malabsorption of vitamin A and
abnormal RBP status:

1.   Malabsorption.

Classically,   malabsorption of fat-soluble vitamins
along with fat was thought to be the primary cause of
vitamin A deficiency in disease states. A given disease
state can interfere with the absorptive process at a number
of stages (Figure 2.3 section i.5):

   a. Dietary intake of vitamin A.

   b. Dietary intake of fat.

   c. Pancreatic lipase secretion.

   d. Cholesterol esterase secretion.

   e. Intraluminal conjugated bile salt concentration.

   f. Synthesis of chylomicrons.

a.   Dietary intake of vitamin A.

Dietary intake of vitamin A may be reduced in any
disease state causing anorexia and/or nausea (Mezey, 1978,
Littlewood & McDonald, 1987).   However, vitamin A
supplementation is routine in such conditions therefore
dietary insufficiency is unlikely to be the precipitating
cause of vitamin A deficiency (Goodchild, 1986, Wesser &
Urban, 1985, Mowat, 1987).

-61-

b.   Dietary intake of fat.

Dietary triglyceride is essential for the absorption of vitamin A (Goodman & Blaner, 1984). Previously, low fat diets have been utilised in order to control steatorrhoea, and this might result in reduced absorption of vitamin A and particularly carotenoids (Goodchild, 1986, Weser & Urban, 1985). However, very low fat diets are now rarely indicated. The use of medium chain triglyceride might also affect vitamin A absorption, since medium chain triglyceride is absorbed via the portal route (Durie et al., 1980) and less dietary fat is therefore available for incorporation into micelles; absorption of vitamin A may be diverted to the less efficient portal route (Forsgren, 1969, Littlewood & MacDonald, 1987).

c.   Pancreatic lipase secretion (I).

Pancreatic secretion of lipase, colipase and bicarbonate is reduced in at least 85% of patients suffering from cystic fibrosis (Mearns, 1985). Although administration of pancreatic supplements improves fat absorption (Harris et al., 1955), significant intestinal malabsorption may still occur (Littlewood & McDonald 1987). Fredrickson and Blackberg (1980) suggested that lingual lipase partially compensates for pancreatic exocrine insufficiency, but control of malabsorption is rarely complete.

Earlier studies demonstrated pancreatic exocrine insufficiency in chronic liver disease associated with alcohol abuse and malnutrition. However, more recent studies suggested that pancreatic exocrine insufficiency was not associated with chronic liver disease (Moeller et al., 1974), although it can occur secondary to chronic malnutrition (Weber & Roy, 1972).

The action of pancreatic lipase is facilitated by conjugated bile salts (Friedman & Nylund, 1980), and the concentration of the bile salts may be reduced in liver disease (Mezey, 1978) or short bowel syndrome as discussed below (Weser & Urban 1985). However, this requirement is not absolute, and colipase may enhance lipase activity in the absence of conjugated bile salts (Borgstrum, 1985). Extensive loss of bowel may also be associated with a reduced number of sites for cholecystokinin and secretin release and therefore, with reduced hormonal stimulation of pancreatic exocrine secretion (Weser & Urban, 1985).

d.   Cholesterol esterase secretion (II).

Little is known about the effect of disease state on secretion of cholesterol esterase; presumably similar effects to those on pancreatic lipase would be expected (see above). Conjugated bile salts facilitate the hydrolytic activity of the cholesterol esterase in the intestinal lumen in preference to esterase activity, therefore the presence

of bile salts allows hydrolysis of vitamin A esters prior to incorporation into the micelle (Freidman & Nylund, 1980).

e.   Conjugated bile salt concentration (III).

The luminal concentration of conjugated bile salts may be reduced by a number of factors in a given disease state:

Excessive bile acid losses in faeces resulting in a reduced bile acid pool have been well documented in cystic fibrosis (Weber et al., 1973). Although the losses are reduced by administration of pancreatic supplements, the reduction in the bile acid pool is still thought to contribute significantly to fat malabsorption (Littlewood, 1987).

The bile acid pool has also been found to be reduced in chronic liver disease (Badley et al., 1970) , either as a result of a reduction in synthesis of cholic acid, or increased excretion of bile salts (Mezey, 1978) In these circumstances, the luminal concentration of conjugated bile salt is inadequate, and micelle formation is reduced. Alternatively, the ratio of the various bile salts might be altered, and this might affect the overall activity of the conjugated bile salts within the intestinal lumen (El-Gorab & Underwood, 1973). In extra-hepatic biliary atresia (EHBA) hepatic secretion of bile salts may not return to normal for 6-12 months following a sucessful Kasai operation (as defined in chapter 3.i) (Lily & Javit, 1976).

Abnormal bowel flora, whether associated with a disease state, or resulting from prolonged use of antibiotics may result in premature deconjugation of the bile salts (Lowosky & Walker, 1969, Martini et al., 1957, Kobayashi et al., 1988). This reduces intraluminal concentration of conjugated bile salts, and may result in the inability to reach the critical micellar concentration. Moreover, treatment of pruritis associated with liver disease with cholestyramine, (Mezey, 1979) causes binding of the conjugated bile salts, and may further reduce the bile acid pool (Barnard & Heaton, 1973).

Removal of the ileum causes interruption of the enterohepatic recirculation of bile salts, resulting in a reduced bile acid pool (Hermon-Dowling, 1973). In addition, gastric acid hypersecretion (Weser & Urban, 1985), if associated with short bowel syndrome can also cause deconjugation of bile salts (Harding et al., 1982).

Absorption of vitamin A may occur via the portal route in the absence of bile, however this may be a less efficient route of absorption (Ganguly, 1969, Forsgren, 1969, Murray & Grice, 1961). This is similar to the pattern of vitamin A absorption demonstrated in abetalipoproteinaemia (Yueng & Veen-Baigent, 1972). There is however, no direct evidence of an alternative route for carotenoid absorption (Underwood, 1984).

-65-

f.   Chylomicron synthesis.

In the rare genetic disease abetalipoproteinaemia, chylomicrons are completely absent, therefore vitamin A cannot be absorbed via the normal route (Bishara et al., 1984). However, elevation of serum vitamin A levels is possible with high oral doses of vitamin A and several investigators have suggested that absorption occurs via the portal route (Yueng & Veen-Baigent, 1972, Murray & Grice, 1961).

g.   Summary

In cystic fibrosis, the major factor contributing to malabsorption of vitamin A appears to be pancreatic insufficiency (Littlewood & McDonald, 1987) however, the reduction in bile salt pool also plays a significant role. Conversely, in chronic liver disease the reduction in conjugated bile salts concentration is the most important factor contributing to malabsorption of vitamin A (Badley et al., 1970). In short bowel syndrome, malabsorption is related to the amount, and position of bowel resected. This results in a reduced surface area for absorption, and a reduced bile salt pool, however the relative importance of these two factors is unclear.

2.   Retinol-binding protein status.

Defective synthesis, and/or release of RBP could result in vitamin A deficiency, since transport of vitamin A would be impaired. RBP status may be affected by several disease states, although total absence of RBP has not been reported:

a.   Chronic liver disease.

The effect of liver disease on RBP status was found to be dependent upon the aetiology of the disease. Significant parenchymal disease results in low serum levels of total RBP and transthyretin, presumably reflecting a reduction in the rate of hepatic protein synthesis (Skrede et al., 1975). Smith and Goodman (1971) demonstrated that reduced serum levels of vitamin A, total RBP and transthyretin improved on resolution of acute hepatitis, correlating with improvement in standard liver function tests.

The low serum total RBP levels in chronic liver disease were found to be associated with reduced dark adaptation (Vahlquist et al., 1978). Intramuscular water-miscible vitamin A did not result in an increase in serum total RBP levels, or an improvement in dark adaptation, indicating that synthesis or release of RBP was impaired in liver disease. Cirrhosis was found to be associated with a reduction in vitamin A and RBP levels (Brissot et al., 1978), however zinc was deficient only in patients with

-67-

alcohol related cirrhosis (Brissot et al., 1978). These findings might indicate that two separate factors might be involved in the development of low serum total RBP levels in cirrhosis.

In alcoholic cirrhosis zinc, an important co-factor in RBP and transthyretin synthesis may be deficient, therefore synthesis of RBP may be reduced (Bates & McClain, 1981, Smith et al., 1973b) . Conversely, Russell and co-workers' (1978) demonstrated an improvement in dark adaptation in alcoholic cirrhosis when vitamin A was administered, indicating that zinc was not associated with the low serum total RBP levels, and that hepatic release of RBP was stimulated by the vitamin A. In non-alcoholic cirrhosis low levels of serum total RBP might be associated with a separate defect in RBP synthesis or release. Zinc is also necessary for the utilisation of vitamin A in the eye (Bridges 1984).

b.   Cystic fibrosis.

Low serum levels of total RBP have been demonstrated in cystic fibrosis despite vitamin A supplementation (Smith et al., 1972, Palin et al., 1979, Underwood & Denning 1972). Hepatic vitamin A stores appeared to be normal in these patients indicating absorption of vitamin A was adequate, but that vitamin A transport might be impaired (Smith et al., 1972); absorbed vitamin A would therefore accumulate in

-68-

the liver, unable to reach sites of action. Serum total RBP levels were reduced in all patients regardless of hepatic status, however some correlation with hepatic function was observed. It therefore remained unclear whether abnormalities in the RBP transport system are primary or secondary features of cystic fibrosis.

c.   Malabsorption and steatorrhoea.

Vahlquist and co-workers (1978) demonstrated a reduction in serum total RBP levels in some, but not all patients with malabsorption. Conversely, Smith and Lindenbaum (1974) found normal serum total RBP levels in malabsorption. RBP status therefore appears to be dependent on the nature, severity and duration of the disease state causing malabsorption.

d.   Malnutrition.

Protein-energy malnutrition may result in reduced serum RBP and pre-albumin levels. Two major factors may contribute to the low serum concentrations of these proteins. Firstly, lack of substrate (amino-acids from dietary protein) and energy (calories) could result in reduced hepatic protein synthesis (Smith et al., 1973a, Arroyave et al., 1961, Ingenbleek et al., 1975). Secondly, intake of vitamin A might be inadequate (Large et al., 1980). The overall effect in an individual would depend on the severity of the protein-energy malnutrition, and the level of dietary

-69-

vitamin A intake. In addition, RBP status might be further influenced by other factors such as nutritional intake of zinc, or the presence of infection.

e. Miscellaneous conditions affecting retinol-binding protein status.

Serum levels of total RBP were also reduced in infection (Goodman, 1984b), burns (Moody, 1982) and multiple trauma (Goodman, 1984b). Serum total RBP levels returned towards normal, following recovery from infection or burns. However, it is not clear whether this fall in serum levels resulted from reduced availablity of vitamin A or reduced synthesis/release of RBP.

Elevated serum vitamin A and total RBP levels were demonstrated in women taking oral contraceptives, the oestrogen component being largely responsible for the increased levels. A bicyclic variation in serum total RBP levels was also demonstrated during the menstrual cycle (Vahlquist et al., 1979).

f. Kidney disorders.

Since the kidney is the major site of RBP catabolism, functionally significant renal disease results in increased serum levels of total RBP. These high levels of serum total RBP are associated with elevated serum vitamin A levels (Smith & Goodman, 1971).

3.   Conclusions.

Vitamin A deficiency is likely to develop as a result
of both malabsorption and defective RBP synthesis. The
relative importance of these two factors is dependent on the
nature and severity of the disease state.

iv) Systemic effects of vitamin A.

Vitamin A is required for night vision, differentiation
and   integration   of   epithelial   tissue,   growth   and
reproduction (Underwood, 1984). Robrigues and Irwin (1972)
suggested that individual tissues demonstrate different
requirements for vitamin A. The daily requirements in
infants aged 0-24 months were estimated as 350-1,100iu to
maintain growth and resistance to infection, whilst 150-
350iu was necessary to sustain dark adaptation and 1,200iu
was necessary to maintain serum vitamin A levels:

1.   in the retina.

The cis-isomer of vitamin A aldehyde is a component of
the visual pigment rhodopsin, which is found in the retinal
rods. Since rods function in dim light, vitamin A is
associated with dark adaptation (Wald, 1960, Bowman & Rand,
1982).

Rhodopsin consists of a protein, opsin, conjugated with
11-cis-vitamin A aldehyde (retinal). Exposure to light
causes isomerisation of the conjugated group, resulting in

formation of all-trans vitamin A aldehyde and opsin (Figure 2.6).

$$
\begin{array}{c}
\text{Rhodopsin} \\
\text{(11-cis vit A + opsin)}
\end{array}
$$

light

cis-vit A   opsin   +   all-trans vit A

dark

Pigment cell
CRBP-vit A
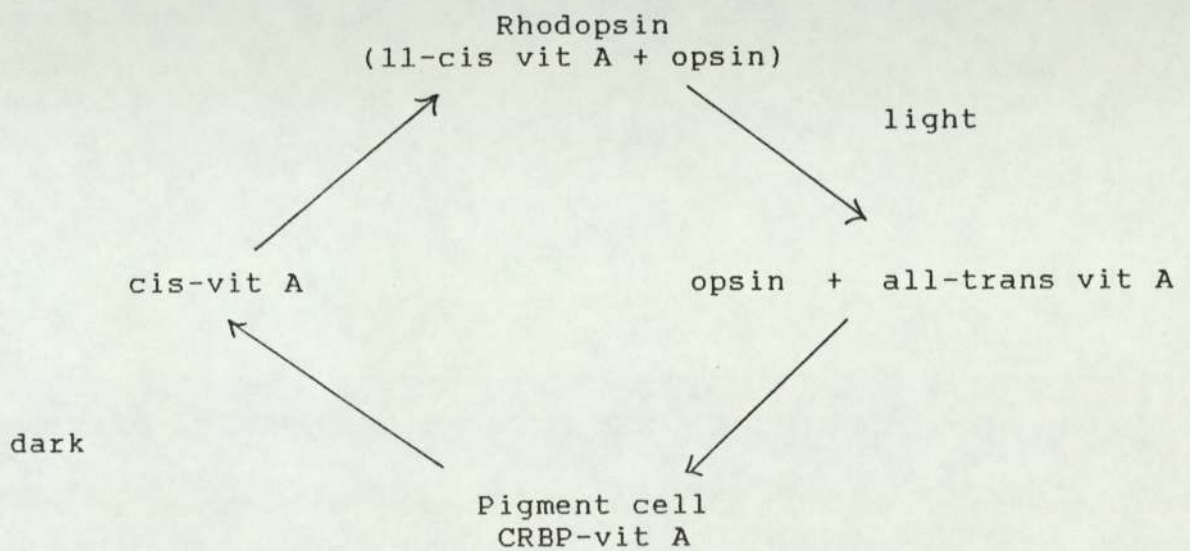
Figure 2.6. represents the role of vitamin A in the visual cycle, under conditions of light and dark.

The all-trans vitamin A aldehyde passes to adjacent pigment cells, probably bound to cellular retinol-binding protein (CRBP). In the absence of light, all-trans-vitamin A aldehyde is converted into the cis-isomer, and transported to the rods, where it combines with opsin. In the light adapted eye, pigment cells contain a high concentration of vitamin A esters (mainly the palmitate and stearate), with little or no rhodopsin in the rods. In the dark adapted eye, the concentration of vitamin A esters in the pigment cells falls, as the concentration of rhodopsin in the rods increases. In conditions of dietary insufficiency the pigment cell probably acts as a reserve for vitamin A (Bridges et al., 1982).

2. in epithelial cells.

Vitamin A is involved in epithelial cell differentiation and proliferation, however the mechanism of action is not fully understood (Wolbach & Howe, 1925). DeLuca (1977) demonstrated that mannitose retinyl phosphate was involved in the synthesis of the cell membrane of glycoproteins, which may play a key role as receptors for specific hormones (Weber, 1983). Conversely, Sporn and Roberts (1983) suggested that retinoids alter the genomic expression of target cells, the genes affected being those which influence differentiation and proliferation. Cellular retinol-binding protein may play a critical role in facilitating interaction of vitamin A with binding sites

-73-

within the cell nucleus (Chytil & Ong, 1979), however retinoids also affect differentiation in cells which do not contain cellular retinol-binding protein (Sporn & Roberts, 1983).

Vitamin A is active in the epithelial surfaces of the conjunctiva, sclera, cornea, skin, and the respiratory, gastrointestinal and urogenital tracts.

v) Manifestations of vitamin A deficiency.

Classically, vitamin A deficiency is associated with impaired dark adaptation and ultimately blindess, although Moore (1957) recognised the diverse pathological effects of vitamin A deficiency. In early investigations, xerophthalmia (see below) was the outstanding feature of vitamin A deficiency, however deficiency was soon found to be associated with widespread damage of mucous membranes, resulting in an increased susceptibility to infection (Chandra, 1988) and poor growth (West et al., 1988).

In the eye alone, vitamin A deficiency can cause damage by:

a.   Prevention of the formation of rhodopsin.

b.   Inadequate provision of vitamin A to nervous tissue, which may cause constriction or twisting of the optic nerve.

c.   Xerophthalmia.

The abnormalities associated with vitamin A deficiency have been reviewed by Moore (1957). As previously discussed, tissues might have different requirements for vitamin A, therefore vitamin A deficiency does not uniformly affect all tissues at a given time, or to the same extent (Robrigues & Urwin, 1972).

The liver contains extensive stores of vitamin A which must be depleted before signs of vitamin A deficiency become apparent, even if vitamin A is almost completely excluded from the diet (Sauberlich et al., 1974, Hume & Krebs, 1949). Hepatic stores of vitamin A may vary considerably in a healthy adult, therefore there is considerable variation in the time it takes for vitamin A deficiency to develop, ranging from 20 to 600 days (Robrigues & Irwin, 1972, Hume & Krebs, 1949). Since hepatic vitamin A stores are poor at birth, young children are more susceptible to vitamin A deficiency (Olson et al., 1984).

1. Ocular signs of vitamin A deficiency.

Ocular signs of vitamin A deficiency have been classified by the WHO (1982) under the general term xerophthalmia; the classification has been given below in parentheses. Signs other than night blindness occur as a result of abnormal differentiation of epithelial cells (Sandford-Smith, 1988).

a.   Night blindness (XN).

One of the earliest signs of vitamin A deficiency is night blindness which is quite specific to vitamin A deficiency in children under the age of 9 years, however specificity is lost as age increases (Genest et al., 1967). Initial loss of rhodopsin is followed by degeneration of the outer segment and loss of photoreceptors. The cause of the degeneration has not been fully explained, however poor stability of opsin may be involved (Bridges, 1984).

b.   Conjunctival xerosis (X1A).

Conjunctival xerosis or drying, results in loss of lustre of the conjuntiva, which may appear thickened, wrinkled and slightly pigmented; xerosis occurs despite adequate tear fluid. Dohlman and Kalevar (1972) suggested that the tear fluid loses stability, since mucus secretion is poor.

c.   Bitot's spots (X1B).

As vitamin A deficiency progresses, xerosis may be associated with Bitot's spots (foamy accumulations of sloughed cells).

d.   Corneal xerosis (X2).

Xerosis of the cornea follows conjunctival xerosis. Initial localised areas of dryness become more extensive, followed by thickening and finally ulceration (WHO, 1982).

e.   Corneal ulceration (Keratomalacia) (X3A and X3B).

Corneal ulceration may result in irreversible
blindness. The exact pathophysiology is unknown, but the
initial step would appear to be damage to the epithelium,
possibly as a result of localised drying, followed by
destructive enzymatic action (Dohlman & Kalevar, 1972).

f.   Xerophthalmic scars (XS).

Despite healing of corneal ulcers, severe scarring is
likely and this may partially or totally obstruct vision.

2.   Non-ocular signs of vitamin A deficiency.

Underwood (1984) summarised the non ocular signs of
vitamin A deficiency, which can be listed as follows:

a.   Increased cerebro-spinal fluid (CSF) pressure.

b.   Anorexia.

c.   Reduced weight gain during periods of growth.

d.   Abnormalities in sensory perception.

e.   Abnormal balance and immune responses.

f.   Increased susceptibility to infection.

g.   Impaired iron metabolism.

h.   Disturbances in the reproductive system.

Many of these signs might be present in subclinical
vitamin A deficiency as described in chapter 1.i. (WHO,
1978).

a.   Increased CSF pressure.

Increased CSF pressure is a rare complication of chronic vitamin A deficiency (Corey & Hayes, 1972, Moore, 1957), which may present as a bulging fontanelle in human infants (Abernathy, 1976).

b.   Growth

Vitamin A deficiency usually occurs during periods of growth in the life cycle (Underwood, 1984). Once adulthood is reached it is difficult to induce vitamin A deficiency (Hume & Krebs, 1949), presumably since hepatic vitamin A stores are adequate (Hume & Krebs, 1949). The association of vitamin A deficiency with a reduced growth rate is well established in animal studies (Moore, 1957), and has been documented in young children (West et al., 1988). Animal studies in sterile conditions demonstrated that the reduction in weight gain was not secondary to subclinical or clinical infection (Beaver et al., 1961, Bieri et al., 1969).

c.   Differentiation and integrity of epithelial and mesenchymal tissue.

Keratinisation of the epithelial tissue in the eye, respiratory, gastrointestinal, and urogenital, tracts can occur in vitamin A deficiency (Wolbach & Howe, 1925). The keratinising metaplasia of mucus-secreting epithelial cells

-78-

is not common to all epithelial tissue (Olson, 1972), and species variation occurs (Logan, 1972). A reduction in goblet cells is observed in mucus secreting epithelium which does not normally keratinise, such as the intestinal mucosa; the loss of protective mucus secretion compromises the surface integrity of the epithelium (Deluca et al., 1969).

Epithelium that contains no goblet cells, such as skin epithelium becomes thickened, dry and scaly (hyperkeratinised) (Maltoltsy, 1976). Epithelium containing both goblet cells and keratinising cells such as the conjunctiva and the cornea (Somer & Green, 1982), respiratory, genital tracts (Wolbach & Hume, 1925), salivary glands and the trachea (Anzano et al., 1980) undergoes hyperplasia and keratinisation in response to vitamin A deficiency. The changes in the cornea and conjunctiva are particularly well documented, with keratinisation and disappearance of the goblet cells (Amedee-Manesme et al., 1988, Dohlman & Kalevar 1972, Sullivan et al., 1973). The mitotic rate of the epithelial cells also increases and Rao and co-workers (1987) suggested that this hyperproliferation precedes the clinical conjunctival changes. However, the stimulus for the changes remains unclear (Rao et al., 1987).

d.   Bone formation and the nervous system.

Chronic vitamin A deficiency might result in the inappropriate deposition of thickened, less dense bone. Hayes & Cousins (1970) postulated that there is an altered

-79-

balance between the number, and activity of osteoblasts and osteoclasts. The role of vitamin A in these processes is not known, however these adverse effects of vitamin A deficiency only seem to occur in growing animals.

Neurological lesions associated with vitamin A deficiency have been described (Moore, 1957). Degeneration in the nerve bundles in the optic thalamus, the optic, femoral and sciatic nerves, along with certain parts of the spinal cord have been noted.

e.   The reproductive system and foetal development.

Vitamin A is required in the reproductive system for the maintenance of differentiated epithelium, support of spermatogenesis in males, and completion of full gestation and embryonic development in females (Moore, 1957, Bates, 1983). Retinoic acid can fulfill the role in epithelial tissue (section i.1.), but vitamin A is required for the other functions.

The developing foetus is dependent on placental transport for vitamin A. The transport mechanism is not fully understood, but appears to involve maternal holo-RBP (see section ii.3a.) (Underwood, 1984).

vi) Hypervitaminosis A.

Hypervitaminosis A or vitamin A toxicity is not considered to be a public health problem, however there is growing concern that increased self-medication in the western world, may result in chronic ingestion of doses in excess of 10-20 times the recommended daily intake, causing vitamin A toxicity (Goodman, 1984a). Toxicity has been reported in subjects taking high dose retinoids·for chronic skin conditions, and a number of cases of toxicity have· also been reported in young children in association with high dietary intake of vitamin A (Lippe et al., 1980, Persson et al., 1965, Stirling et al., 1980) .

Furthermore, if high doses of vitamin A were administered to patients, unable to transport the vitamin as a result of defective RBP synthesis or release, toxicity might occur. In the absence of RBP, the concentration of free vitamin A esters circulating in serum might increase, and the unregulated delivery of vitamin A to sites of action might cause toxicity (Smith & Goodman, 1976, Vahlquist et al., 1982). In addition, absorption and transport of vitamin A might increase if the clinical condition of a patient improved. In these circumstances the continued administration of high doses of vitamin A might result in toxicity.

The signs of hypervitaminosis A are diverse (Goodman, 1984a), but may include raised intracranial pressure, headache, nausea, vomiting, and skeletal pain (Goodman, 1984a). Hepatomegaly, hypercalcaemia and haematological abnormalities occur less frequently (Stewart-Trusnell, 1985). Considerable information is available about the toxic effects of vitamin A on tissues, and a detailed review has been provided by Benedich and Langseith (1989). Smith and Goodman (1976) suggested that toxicity occurs in a healthy individual when saturation of RBP is exceeded, and vitamin A esters circulate in association with lipoproteins in the serum, resulting in non-specific and unregulated delivery of vitamin A to membranes.

PART II : METHODOLOGY

CHAPTER 3 : SUBJECTS, SPECIMEN COLLECTION AND STORAGE.

i)   Subjects.

The subjects for the present study were recruited from
patients attending routine out-patient appointments at The
Children's Hospital, Birmingham. Hospital staff volunteers
acted as adult controls (25.00$\pm$0.70 yrs) (n=9).

1.   Measurement of serum vitamin A levels.

Serum specimens were obtained from 182 patients with
liver disease, cystic fibrosis or gastrointestinal disease,
who required a routine blood sample.

Owing to variation in the practice of different medical
teams,   patients   with   chronic   liver   disease   and
gastrointestinal disease were receiving a standard oral
daily vitamin A supplement of 2,500iu (Ketovite Liquid),
whilst patients with cystic fibrosis received a different
vitamin   supplementation   regimen;   all   infants   were
supplemented with 8,000iu of vitamin A, (1.2ml Abidec
drops), older patients < 30kg received 2,500iu of vitamin A
(multivitamin capsules B.P.C), whilst those > 30kg received
5,000iu of vitamin A (multivitamin capsules B.P.C.).

a.   Patients with chronic liver disease.

A brief summary of the nature of the liver diseases
encountered in the present study has been included in the
glossary of terms. Of the 90 patients (aged 0.08 to 18
years) who attended the liver clinic, 31 suffered from

extra-hepatic biliary atresia (EHBA) (A successful Kasai[*]
operation had been performed in 14 of the children), and 20
had cirrhosis. The diagnoses for patients with cirrhosis,
and the remaining 42 patients with non-cirrhotic chronic
liver disease have been given in **Tables 3.1** and **3.2**,
respectively.

Diagnosis	(n)	Age Mean (SEM) (years)	Age Range (years)
Cystic fibrosis	(3)	16.00 (1.50)	13.00-18.00
Chronic active hepatitis	(2)	13.13	12.25-14.00
Idiopathic cirrhosis	(13)	2.72 (1.18)	0.25-10.00
Sclerosing cholangitis	(2)	8.29	3.83-12.92

Table 3.1. Patients with cirrhosis (n=20).

b.   Cystic fibrosis.

A further 78 patients had cystic fibrosis (58 children
aged 8 to 18 years and 20 young children aged 1 to 4 years).
All patients were receiving enteric coated microsphere
pancreatic supplements, either Creon (Duphar Laboratories
Ltd, Southampton) or Pancrease (Ortho-Cilag Pharmaceutical
Ltd, High Wycombe) capsules. These preparations have been
reported to be equally effective in improving fat
absorption, and the dose was titrated to meet the

-86-

Diagnosis	(n)	Age Mean (SEM) (years)		Age Range (years)
Neonatal hepatitis	(11)	0.29	(0.07)	0.04-0.63
Cholestasis**	(3)	0.56	(0.04)	0.06-0.63
alpha$_1$-Antitrypsin deficiency	(5)	1.58	(0.45)	0.25-3.17
Glycogen storage disease	(4)	7.55	(1.67)	6.17-12.25
Crigler-Najjar syndrome	(2)	7.12		5.67-8.75
Alagilles syndrome	(4)	8.70	(3.60)	2.00-15.67
Wilson's disease	(1)	4.00		
Abetalipoproteinaemia	(1)	4.50		
Liver cysts	(1)	0.29		
Hyperlipidaemia	(2)	13.71		11.67-15.75
Post liver transplant	(8)	3.64	(1.67)	0.83-13.67

** Cholestasis had been associated with parenteral nutrition, and was resolving.

Table 3.2. Patients with non-cirrhotic chronic liver disease (n=42).

* A Kasai operation was defined as successful if bile drainage was achieved, and there was no evidence of progressive cirrhosis. It is possible to achieve bile drainage however, progressive cirrhosis may result in further morbidity & mortality. In these cases, although the operation might be technically successful prognosis was poor.

individual's requirements (Beverley et al., 1987, Robinson et al., 1989). None of the patients had clinical evidence of hepatic involvement, the 3 patients with cystic fibrosis and known cirrhosis have been included in the group of patients with chronic liver disease (see Table 3.1).

c.   Gastrointestinal disease.

The remaining 23 patients (aged 2 months to 14 years) had gastrointestinal disease (Table 3.3), and all except those being investigated for undiagnosed malabsorption, anorexia, and weight loss, or Hirschsprung's disease were receiving appropriate treatment. Patients who were failing to thrive or had a short bowel were receiving increased calorific intakes, whilst the patients with coeliac disease were all maintained on a gluten free diet. The patient with pancreatic exocrine insufficiency was receiving Pancrease capsules as a pancreatic supplement.

2.   Absorption tests.

An absorption test for vitamin A was completed in 9 patients who had persistently low serum vitamin A levels $(0.32\pm0.05\mu mol/l)$ (n=14). A single 5,000iu dose of vitamin A (as Ketovite liquid) was administered to 5 of the patients (3 EHBA, 1 neonatal hepatitis, 1 $alpha_1$-antitrypsin deficiency) and 5 adult controls, whilst a further 3 patients (1 EHBA, 1 idiopathic cirrhosis, 1 ultra-short bowel) received a single dose of 10,000iu of vitamin A (as

-88-

Arovit Drops). A dose of 100,000 of vitamin A (as Arovit) was administered to the final patient with abetalipoproteinaemia. The absorption of vitamin A was also determined in the presence of 3g vitamin E in the last patient.

Diagnosis	(n)	Age Mean (SEM) (years)	Age Range (years)
Failure to thrive	(11)	1.45 (0.36)	0.17-3.58
Coeliac disease	(3)	0.72 (0.15)	0.50-1.00
Malabsorption*	(3)	9.89 (2.16)	6.67-14.00
Hirschsprungs disease	(1)	2.58	
Short bowel syndrome	(4)	4.69 (2.87)	0.75-13.00
Pancreatic exocrine insufficency	(1)	0.50	

* Subjects were being investigated for malabsorption, anorexia and weight loss.

Table 3.3. Patients with gastrointestinal disease (n=21).

3. Measurement of serum total retinol-binding protein levels.

Specimens for determination of serum total retinol-binding protein were obtained from 41 patients with chronic liver disease or cystic fibrosis, on 62 separate occasions. These were compared with 11 specimens from 6 liver transplant recipients, and 5 specimens from healthy adult

-89-

Diagnosis	(n)	Male/ Female	Age Mean (SEM) (years)	Age Range (years)
Cirrhotic liver disease				
EHBA	(16)	5/11	1.69 (0.34)	0.08-4.00
Cirrhosis	(6)	4/2	1.70 (0.16)	1.42-2.17
Sclerosing cholangitis	(1)	0/1	5.92	
Congenital fibrosis	(1)	0/1	1.83	
Primary biliary cirrhosis	(2)	1/1	49.50	46.00-53.00
Non-cirrhotic liver disease				
Neonatal hepatitis	(4)	3/1	0.30 (0.13)	0.08-0.67
alpha$_1$-Antitrypsin deficiency	(1)	1/0	0.83	
Wilson's disease	(1)	1/0	4.00	
Abetalipoprotein- aemia	(1)	1/0	5.00	
Cystic fibrosis	(10)	5/5	4.59 (1.99)	1.00-18.00
Controls				
Post-transplant	(6)	4/2	4.85 (2.3)	1.08-14.00
Adult controls	(5)	2/3	25.50 (0.71)	24.00-28.00
Malabsorption	(3)	3/0	9.89 (2.16)	0.33-1.33
Hypercarotenaemia	(2)	0/2	40.00	38.00-42.00

Table 3.4. Patients for whom serum retinol-binding protein was determined (n=59).

controls. Specimens were also obtained from 3 patients with malabsorption (but not liver disease), and 2 adults with hypercarotenaemia. Serum vitamin A levels were determined for all specimens. Details of the patients have been given in Table 3.4.

ii) Specimen collection & storage.

Clotted venous blood was collected in plain tubes containing no heparin or EDTA, at least 8 hours after administration of the last vitamin A supplement. Specimens were centrifuged for 2 minutes at 2500rpm, before serum was decanted and stored at $4^{\circ}C$ for not more than 7 days prior to vitamin A analysis. Delayed analysis of serum total retinol-binding protein was anticipated in the present study, therefore specimens were stored at $-20^{\circ}C$ for up to 6 months, since total retinol-binding protein has been shown to be stable under these conditions (Dawson, 1986). All specimens were protected from light, throughout storage and during analysis where possible.

iii) Specimen volume.

The optimum specimen volume for vitamin A analysis was 1.0ml (Neeld & Pearson, 1963), however volumes as small as 0.2ml have been utilised (personal communication, Cowen). Specimens Of 0.4-0.9ml were treated in the same way as a larger specimen, however specimens of 0.3ml or less had to be diluted with petroleum ether to produce sufficient

-91-

supernatant (0.9ml) for analysis. A further 0.1ml of serum was required for the retinol-binding protein assay.

iv) Statistical analysis.

All data has been expressed as Mean±SEM with the number of specimens given in parenthesis. Where the number of specimens was < 3 no SEM has been shown. In many cases more than one specimen was obtained from each patient for determination of serum vitamin A levels, and all of these specimens were included in the analysis. This should allow for the expected fluctuation in serum vitamin A levels within an individual (Underwood, 1984). However, this method of analysis resulted in a possible source of bias, therefore the results were recalculated by obtaining a mean serum vitamin A level for each patient prior to statistical analysis of the group results. Since this did not significantly affect the findings of the present study, the results were expressed as calculated using the former methodology.

Differences between groups of data, were assessed using the unpaired Student's t-test, except where distribution of the data was known to be skewed. The Mann-Whitney test was therefore applied to the non-parametric data obtained from the absorption tests, liver function test and calculation of caffeine clearance.

Pearson's Coefficient was used to determine correlation between groups of data with a normal distribution, whereas Kendalls rank correlation was applied to data with a skewed distribution, such as the data from the liver function and caffeine clearance tests.

P values < 0.05 were taken to indicate significance for both tests of significance and correlation.

Calibration curves for vitamin A and carotene were determined using Demings linear regression.

v)   Drugs used in the study.

Drug	Supplier
Arovit Drops containing 5000iu	Roche Products Ltd,
vitamin A/drop (as palmitate)	Welwyn Garden City.

Vitamin A Solution (Type 100)

containing 100,000iu vitamin A

in 1ml (as palmitate)

Ro-A-Vit Tablets containing

50,000iu vitamin A (as acetate)

Vitamin E Suspension containing

500mg α-vitamin E acetate in 5ml

Vitamin E Injection (Ephynal)

containing 100mg α-vitamin E

acetate in 1ml

Abidec Drops containing 4,000iu   Warner Lambert Health
vitamin A in 0.6ml (as palmitate)   Care Ltd, Eastleigh,

                                         Hants.

Ketovite Liquid containing   Paines & Byrne,

2,500iu vitamin A in 5ml   Greenford, Middlesex.
(as palmitate)

Multivitamin Capsules B.P.C.   Evans Medical Ltd,

containing 5,000iu vitamin A   Dunstable, Beds.

(as palmitate)

vi) Materials used in the study.

1.   Measurement of serum vitamin A levels.

     Chemical   Supplier
Absolute   Alcohol   James Burrough Ltd,

                                          London.

Petroleum Ether   Analar, BDH, Poole.

Trifluoroacetic Acid

Chloroform

Beta-Carotene[*]   Roche Products Ltd,

(RO8-8427)   Welwyn Garden City.

Crystalline All-E-Retinol[*]

(RO 01-45955)

[*] A kind gift from Roche Products Ltd.

2.   Measurement of serum retinol-binding protein levels.

Reagents	Supplier
Antibody to Retinol-binding Protein (RBP):	Dako Ltd,
Rabbit immunoglobulin to human RBP	High Wycombe.
Antibody to RBP/Enzyme Conjugate:	
Horse-radish Peroxidase-conjugated	
rabbit immunoglobulins to human RBP	

RBP Standard serum   Behringer

Protein-standard-serum for nor-partigen   Diagnositics,

(RBP   reported as 90mg/l)   Hoechst U.K.

Colour substrate: Ltd, Hounslow

(Tetramethylbenzadine (TMB) in dimethylsulphoxide

(DMSO) (10mg/l) which produces a yellow colour

when broken down by the enzyme conjugate.)

3.   Impression cytology technique.

Chemicals	Supplier
Mixed cellulose esters filters	Millipore (UK)
(MF Millipore Filter VS) 0.22µm	Ltd, Harrow,
pore size, pre-cut into strips,	Middlesex.
approx 5mm wide.	
Cellulose Nitrate filter	Sartorus,
(Type 11305) 0.45µm pore size,	Gottingen,
pre-cut into strips	West Germany.

Absolute Alcohol	James Burrough
	Ltd, London.
0.5% Periodic Acid	BDH, Poole,
Schiff's Reagent	Dorset.
Lithium Carbonate	
Haematoxylin Conterstain (filtered)	Midland
Methanol	Laboratories
Xylene	Lichfield, Staffs
DPX Mountant	Nustain,
	Nottingham.

vii) Solutions prepared for the retinol-binding protein
      assay.

All chemicals used in the preparation of solutions were
analytical grade, and were obtained from BDH Chemicals Ltd,
Poole, Dorset, with the exception of the potassium chloride
and sodium chloride (FSA Laboratory Supplies, (Fisons plc
Analytical Reagents), Loughborough).

Antibody Coating Buffer

Carbonate/Bicarbonate Buffer pH 9.6

1.59g Na_2CO_3

2.93g $NaHCO_3$

in 1 litre Distilled Water

Store frozen, do not keep at room temperature for more than
3 weeks.

"Blocking Buffer"

PBS/BSA 0.1% pH 7.4

8g   NaCl

0.2g KH$_2$PO$_4$

2.9g Na$_2$HPO$_4$.2H$_2$O

0.2g KCl

1.0g Bovine serum albumin (BSA)

in 1 litre of Distilled Water

The buffer was used within 48 hours of preparation.

Washing Buffer

PBS/Tween pH 7.4

Substitute 0.5ml Tween 20 for BSA

This buffer was used within 48 hours of preparation.

Substrate Buffer

0.1M Sodium Acetate Citrate Buffer pH 6.0-6.10

4.1g Na Acetate

in 500ml of Distilled Water

iv)   Apparatus.

1.   Measurement of serum vitamin A levels.

Apparatus	Supplier
Whirlimixer	Fisons Apparatus Ltd, Loughborough, Leics
Unicham Spectrophotometer SP505	Unicham Ltd,

2.   Measurement of serum retinol-binding protein.

Microtiter   Plates: Dynatech Laboratories,

96-well, flat-bottomed, cobalt-   Ltd, Billingshurst,

irradiated immulon plates, type 129B   Sussex.

Plate-reader: Flow Laboratories,

Titertek   Multiskan   Rickmansworth, Herts.

CHAPTER 4 : METHODOLOGY FOR THE ASSAYS EMPLOYED IN THE

STUDY.

Serum

+ petroleum ether

↓

Extraction of vitamin A & carotenoids

↓

Read absorbance of carotenoids at 450nm

↓

Evaporate to dryness and redissolve in chloroform

+ TFA

↓

Vitamin A & carotenoids ⟶ Blue colouration

↓

Read absorbance at 620nm
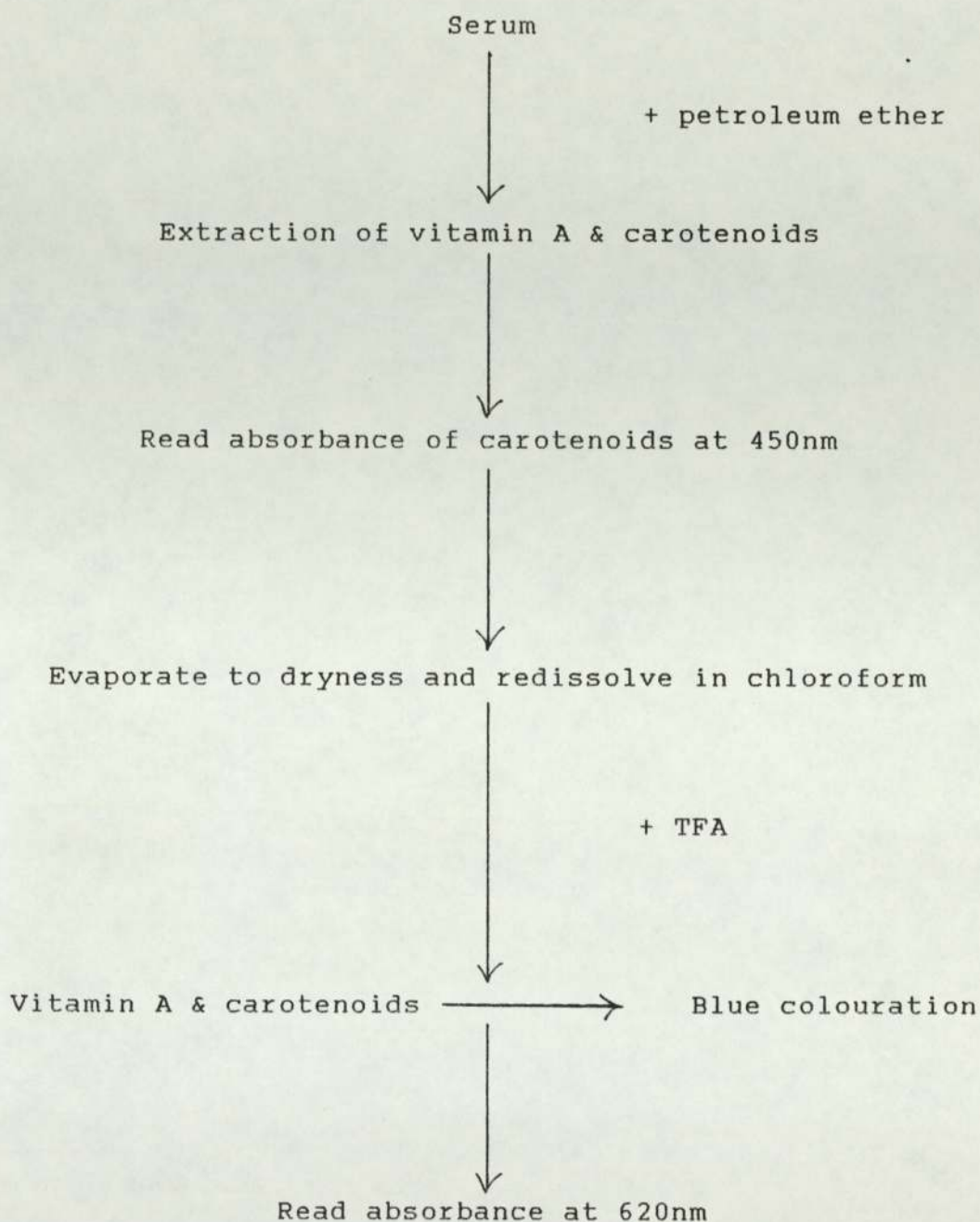
Figure 4.1. represents the principle of the colorimetric
assay for vitamin A & carotenoids. Vitamin A & carotenoids
were extracted into petroleum ether and the absorbance due
to carotenoids at 450nm was read. Vitamin A & carotenoids
both reacted with TFA to form an intense blue colouration,
the absorbance of which was read at 620nm. A correction for
interfering carotenoids was made, using a factor calculated
using a standard calibration curve.

i)   Serum vitamin A levels.

A number of analytical methods are available for
determination   of   serum   vtamin   A   levels,   including
colorimetry (Neeld & Pearson, 1963), high performance liquid
chromatography (HPLC) (Thurnham et al., 1988) or fluorimetry
(Hansen & Warwick, 1969). These methods have been reviewed
by Frolik and Olsen (1984) (see chapter 5.i). At the time of
the present study the colorimetric assay was already
employed at the Birmingham and Midland Eye Hospital,
therefore serum vitamin A levels were determined using this
assay.

1. Principle of the assay.

Serum vitamin A levels were determined using the
modified Carr-Price Reaction (Neeld & Pearson, 1963). In
this method vitamin A, and vitamin A esters react with
trifluoroacetic acid (TFA) to form an intense, transient
blue colouration, the absorbance of which was measured at
620nm (Figure 4.1). The interaction initially involved the
extraction of a hydroxy moiety, leaving a retinylic cation
(λmax 586nm) which formed a complex with trifluoroacetic
acid at the C-4 position (λmax 619nm), or at the C-15
position (λmax 586nm).   Deprotonation of the retinylic
cation, or dissociation of the latter two complexes gave
anhydroretinol (Blatz & Estranda,   1972).   The reaction
pathway has been shown in Figure 4.2. The absorbance of
carotenoids was directly measured at 450nm.

-101-
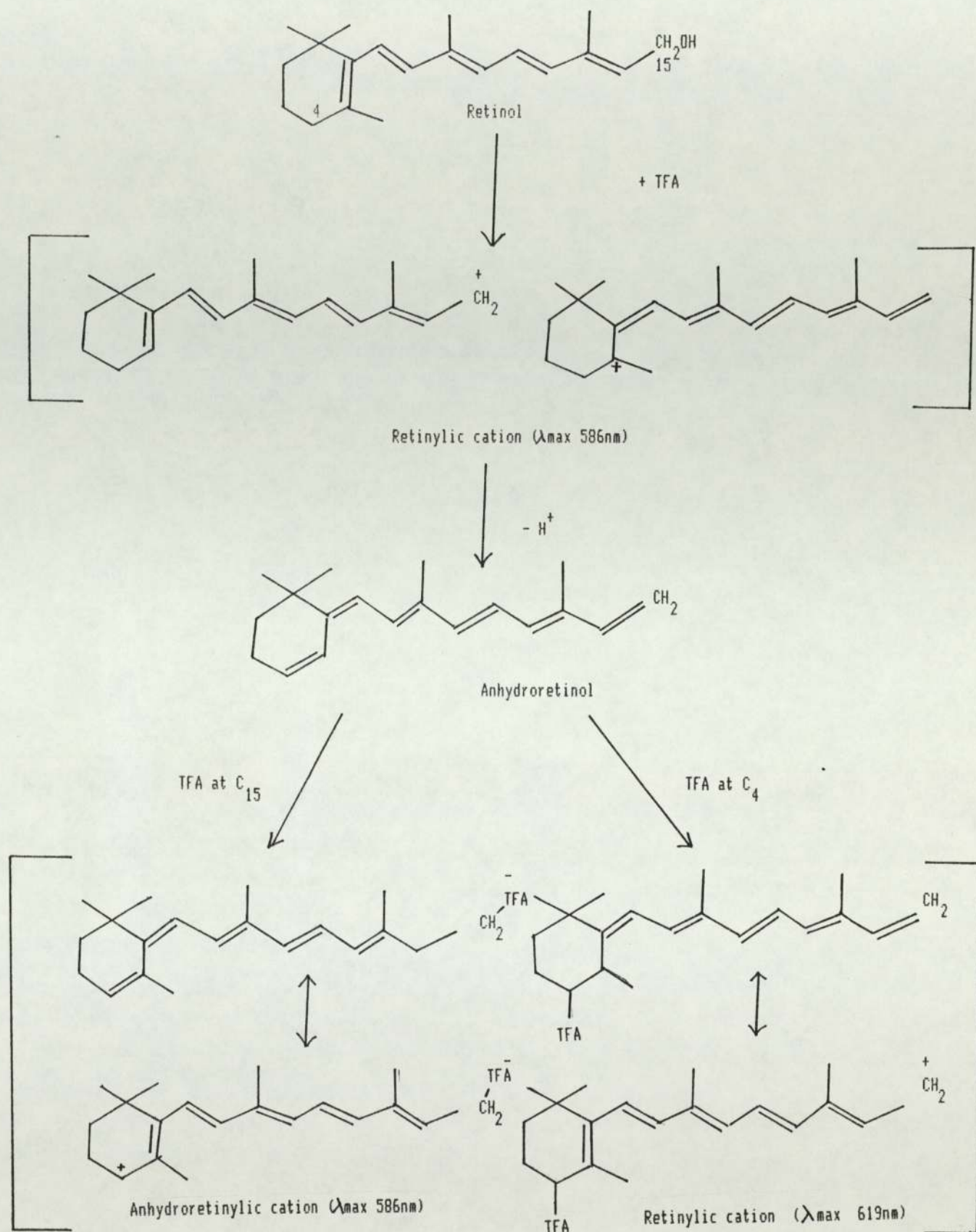
Figure 4.2. represents the reaction pathway in the Carr-Price reaction. A hydroxy moiety has been extracted from retinol (vitamin A), leaving a retinylic cation (λmax 586nm) which forms a complex with TFA at C-4 (λmax 619nm) or at C-15 (λmax 586nm). Deprotonation of the retinylic cation or dissociation of the latter 2 complexes gave anhydroretinol.

2.   Assay procedure

a.   Serum was placed in a stoppered, glass test-tube, and an equal volume of absolute alcohol was added, dropwise, with shaking, to precipitate protein.

b.   Three times the volume of serum (or 1.2ml, whichever was the greater) was added, and the tube contents were carefully vortex-mixed for 2 minutes, before being allowed to settle.

c.   2.4ml of the supernatant (if specimen was less than 0.9ml, supernatant volume would be between 0.9-2.4ml) was pipetted into a cuvette, and the absorbance at 450nm was measured.

d.   The supernatant was then evaporated to dryness at 55°C under vacuum, allowing rapid evaporation without degradation of vitamin A.

e.   The trifluoroacetic acid reagent was prepared by mixing two parts chloroform with one part trifluoroacetic acid, immediately prior to use.

f.   After allowing to cool to room temperature, the sides of the cuvette were washed with 0.2ml chloroform, before 1.0ml of the TFA reagent was added rapidly.

g.   The absorbance at 620nm was measured before the blue colouration began to fade (approximately 30 seconds). A correction was made for any interfering carotenoid (see below).

3. Calculation of serum vitamin A & carotenoid levels.

The carotenoid and vitamin A concentration of each specimen was calculated from the following equation using data obtained from calibration curves (See chapter 5.ii):

$$[Car]_S = A_{450nm} \times 18$$
$$(mg/l)$$

$$[Vit\ A]_S = (A_{620nm} \times 1.2 \times 10^4) - (A_{450nm} \times 18 \times 120)$$
$$(iu/l)$$

For specimens < 1.0ml:

$$A_{620nm} \times \frac{2.4}{supernatant\ volume}$$

To convert iu/l to μmol/l:

Since one iu of retinol is equivalent to 0.3μg, and the molecular weight of retinol is 286, iu/l was converted to μmol/l by:

$$\times \frac{0.3}{286}$$
$$= 0.0010489$$

4. Intra-assay variation (coefficient of variation).

The intra-assay variation (V) was calculated for 10 1.0ml aliquots of pooled serum (mean vitamin A concentration 2.18±0.06μmol/l, mean carotenoid concentration

0.94±0.07mg/l). This was repeated on a second serum pool (mean vitamin A concentration 1.13±0.09μmol/l, mean carotenoid concentration 0.73±0.05mg/l) which had been stored at 4°C for 21 days. The intra-assay variation was calculated according to the following formula:

$$V = \frac{s}{\bar{x}}$$

5.   Calibration of the assay.

Control samples were not included in each batch of specimens, as vitamin A is known to be unstable during storage of serum (Frolik & Olson, 1984). However pure samples of retinol (vitamin A) and beta-carotene were used to calibrate the assay at the beginning of the present study.

a.   Carotene calibration.

A stock solution of 100mg/100ml of beta-carotene in petroleum ether was prepared, and diluted to produce solutions ranging in concentration from 10-140 microgram/100ml. Since 1ml of serum was extracted into 3ml of petroleum ether, the carotenoid concentration of the dilutions had be reduced by one third to produce a calibration curve equivalent to serum carotenoid concentration plotted against absorbance at 450nm (Figure 5.1, chapter 5.ii). A carotenoid correction curve for

-105-

determination of vitamin A was obtained by plotting serum carotenoid concentration against absorbance of calibrant at 620nm (Figure 5.2, chapter 5.ii).

b. Carotene recovery.

A recovery experiment for beta-carotene was performed on a serum pool. The petroleum ether used in the assay procedure was replaced with the equivalent volume of each dilution of the stock carotene solution, used in the calibration experiments.

The absorbance measured at 450nm was the sum of the absorbance of the carotene solution (A_1) and the serum pool (A_2). Therefore:

$$\% \text{ Recovery} = \frac{A_{450nm}}{A_1 + A_2} \times 100$$

All experiments were repeated following storage of the serum pool and the stock carotene solution for 2 weeks at $4^{\circ}C$, both protected from light.

c. Vitamin A calibration.

A solution of vitamin A in chloroform (2×10^5 iu/l) was prepared to calibrate the assay, using a method adapted from Varley and co-workers (1976). The method involved a direct measurement of the absorbance of serial dilutions of the standard vitamin A solution, therefore there was no

allowance for any error resulting from the extraction or evaporation stages of the assay. The absorbance at 450nm of each dilution of the solution of vitamin A in chloroform at 450nm was also determined in order to ensure that vitamin A did not interfere with the determination of serum carotenoid concentration.

Dilutions ranging in concentration from 100-20,000iu/l were produced from the stock solution of $2x10^5$iu/l (60mg/l) vitamin A in chloroform. The absorbance of each dilution was determined at 620nm, as described in the assay procedure. The equivalent serum concentration of vitamin A was calculated as :

$$\frac{\text{concentration of vitamin A in the dilution}}{4}$$

since:

1ml of serum is extracted into 3ml petroleum ether, therefore:

$$2.4\text{ml petroleum ether} \equiv 0.8\text{ml serum}$$

and residue is taken up in 0.2ml of chloroform after evaporation, therefore:

$$0.2\text{ml chloroform} \equiv 0.8\text{ml serum}$$
$$1\text{ml chloroform} \equiv 4\text{ml serum}$$

6.   Investigation of variables associated with the assay.

a.   Haemolysis.

The effect of haemolysis on specimens was investigated by adding known quantities of a haemolysate to a serum pool, as follows:

A pool of red blood cells was washed three times with normal saline to ensure removal of any vitamin A contained in contaminating serum, however traces of vitamin A might have remained on the cell walls. One drop of chloroform and 3ml water were added to the red blood cells in order to produce complete haemolysis, and allow dissolution of the haemoglobin released from the cells. The resultant haemolysate should be vitamin A free. In order to confirm this, duplicates of the haemolysate and a serum pool were assayed for vitamin A and carotenoids.

Volumes of the haemosylate ranging from 1.66 to 10μl were added to the 1ml aliquots (in duplicate) of the serum pool, and these were assayed to determine the effect of haemolysis.

b.   Time of vortex mixing.

The effect of vortex mixing specimens for times ranging from 0 to 5 minutes on the vitamin A content of duplicates a serum pool was investigated, since times of up to 3.minutes had previously been employed (Kaser & Stekol, 1942, Neeld & Pearson, 1963).

c.   Specimen volume.

The effect of specimen volume on the apparent vitamin A and carotenoid content of a serum pool was investigated on duplicate aliquots of 0.2-1.0ml.

d.   Storage of specimens.

A series of experiments was designed to investigate the effect of both short and long term storage of specimens on vitamin A and carotenoid concentration.

I.   Short term storage.

Three separate serum pools were analysed at daily intervals, under storage conditions, ranging from room temperature to $-20^{\circ}C$ for a period of 5 days.

II.   Long term storage.

Specimens (n=41) were stored at $4^{\circ}C$, and $-20^{\circ}C$ for periods of up to 84 days, prior to analysis.

ii)   Vitamin A absorption tests.

1.   Procedure for the absorption tests

a.   Routine vitamin supplements were not administered for at least 24 hr, prior to the test.

b.   Blood specimens were taken prior to, and every 2 hr following the test dose, up to a maximum of 7 hours. A final blood specimen was taken after 24

hours. All specimens were taken via a single heparinised cannula.

c.   All specimens were collected in a plain tube, and were stored as previously described for vitamin A. Serum for determination of vitamin E was stored at $-20^{\circ}C$, since delayed analysis was anticipated.

d.   Serum vitamin A levels were determined as previously described. Serum vitamin E levels were determined using high performance liquid chromatography (HPLC) (Thurnham et al., 1988).

iii) Serum total retinol-binding protein levels.

Several methods are available for the determination of serum retinol-binding protein levels (RBP), including radioimmunoassays (Glover et al., 1974, Baker et al., 1967) and gel chromatography (Peterson 1971). However, work at Aston University in Birmingham has involved the less time consuming double antibody sandwich enzyme-linked immunosorbant assay (ELISA), therefore this assay was employed in the present study (Dawson, 1986). The ELISA assay allows determination of serum total RBP, without separation of apo- (RBP not bound to vitamin A) and holo-RBP (complexed with vitamin A) is not possible.

1.   Principle of the assay.

The principle of the ELISA assay for serum total RBP
has been shown in Figure 4.3 (Dawson, 1986). An antibody to
RBP was adsorbed onto a microtiter plate.   The serum
containing RBP was then added, and this binds to the
antibody. This was followed by the addition of an antibody-
enzyme conjugate which also binds to RBP. A substrate for
the enzyme was then added, which, when broken down by the
enzyme, produces a colour. The amount of colour was
proportional to the amount of RBP present in the specimen.

2.   Assay procedure.

a.   The microtiter plates were coated with $100\mu l$/well of a
      1 in 100 (1.5mg/l) solution of the antibody to RBP in
      carbonate/bicarbonate buffer, prior to storage at 4°C
      overnight, covered with cellophane (Dynatech Inc,
      U.S.A.).

b.   The plates were then washed three times with 200ul
      PBS/bovine serum albumin buffer; plates were left for a
      few seconds before shaking the solution from the wells.
      The third washing solution was left in the wells for 1
      hr at room temperature, to block any sites unoccupied
      by the antibody. The plates were then emptied.

c.   Protein standard serum, which was used as the reference
      standard, and serum specimens were diluted in PBS/Tween
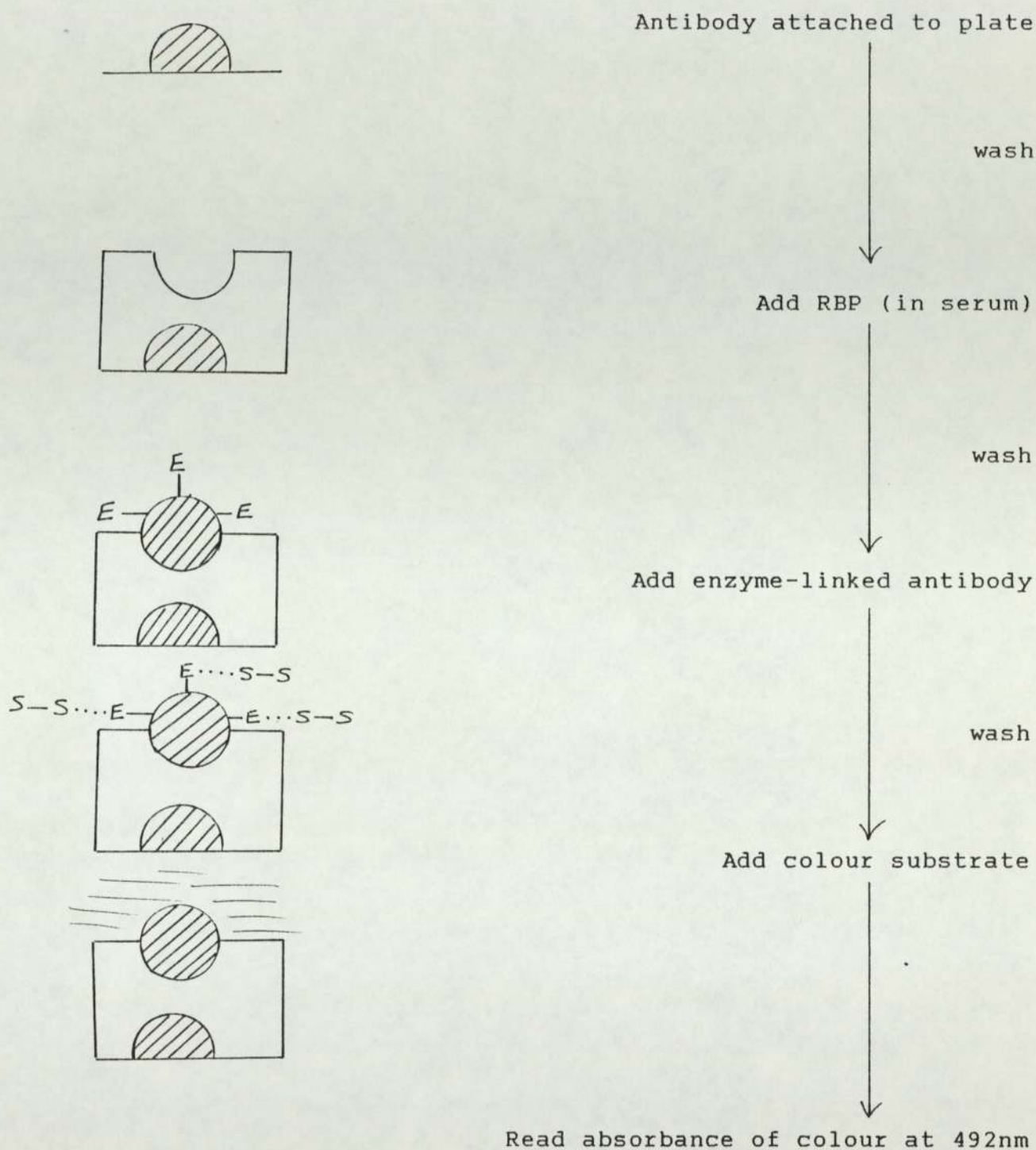
Figure 4.3. represents the principle of the ELISA assay for serum total RBP. An antibody to RBP was adsorbed onto a microtiter plate. Serum was then added and any RBP binds to the antibody. this was followed by addition of an antibody-enzyme conjugate which also binds to RBP. A substrate for the enzyme was then added and this was broken down to produce a colour, the absorbance of which was measured at 492nm.

-112-

buffer. The protein standard serum and serum specimens with a vitamin A concentration >0.50µmol/l were diluted from a 1 in 2,000 (45µg/l total RBP), in doubling dilutions to a 1 in 64,000 dilution (1.5µg/l total RBP). Serum specimens with a vitamin A concentration <0.50µmol/, were diluted from a 1 in 1,000 to 1 in 32,000 dilution, as low serum total RBP levels were anticipated.

d.   Duplicates of each dilution were applied to the plate, 100µl/well. Control wells, containing PBS/Tween were included on each plate. Plates were then covered, and left for 2 hr at room temperature, before washing three times with PBS/Tween.

e.   Once the wells had been emptied, the conjugate was diluted 1 in 300 (0.01mg/l) in PBS/Tween, and 100µl was added to each well. Plates were covered and left for 2 hr at room temperature, before washing 5 times with PBS/Tween.

g.   The substrate solution, was prepared by added the 0.1M citric acid, dropwise to the 0.1M sodium acetate. Meanwhile the Tetramethylbenzadine/Dimethylsulphoxide was allowed to defrost, and 0.5ml was added to 59.5ml of the freshly prepared sodium acetate citrate buffer, followed by 20ul of hydrogen peroxide.

h.   100μl of the substrate solution was applied to each well, and left for 10 minutes at room temperature. 100ul of 2.5M sulphuric acid was then added to each well to stop the colour reaction.

i.   The absorbance of each well was read at 450nm, using the Titertek Multiskan microplate reader.

3.   Calculation of serum total retinol-binding protein levels.

The amount of total RBP present in each specimen was calculated using a calibration curve of the absorbance of different concentrations of the protein standard serum (mean of the duplicates), plotted against $\log_{10}$ concentration of total RBP (mg/l). The calibration curve was calculated using linear regression analysis and a typical calibration curve for the ELISA assay of total retinol-binding protein has been shown in Figure 4.4.

From the absorbance of each dilution of the specimen, the amount of total RBP was read from the calibration curve. This value was then multiplied by the dilution factor to give the amount of total RBP present in the sample. The actual amount of total RBP in the sample was taken as the mean of the values obtained from dilutions of each specimen, which fell within the calibration range.
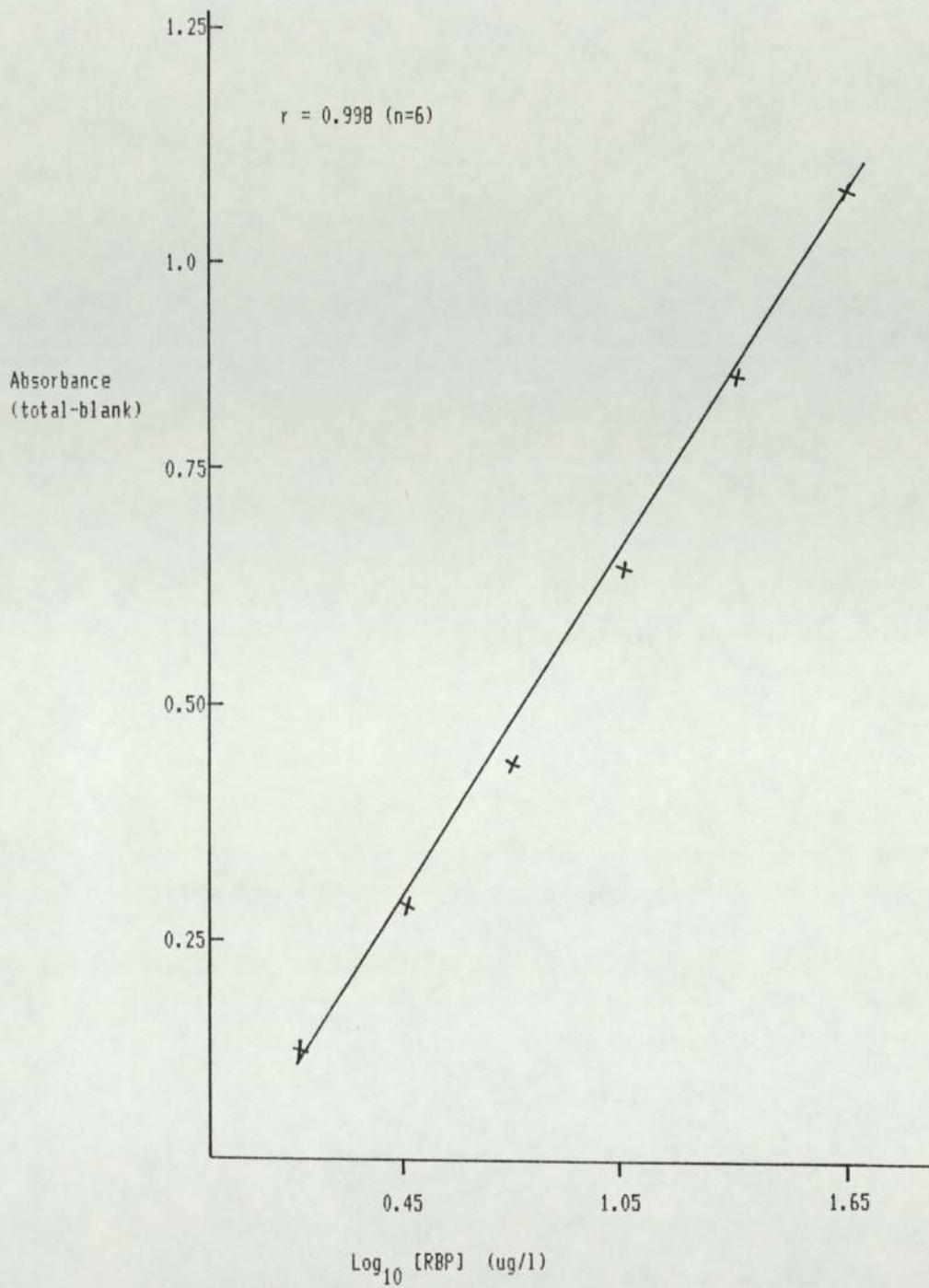
Figure 4.4. represents a typical calibration curve for the
ELISA assay of total RBP using standard serum. (0.45-
1.65µg/l). Correlation coefficient (r) = 0.998, (n=6).

4.   Inter-assay & intra-assay variation.

Determination of serum RBP was repeated on 19 specimens in two separate assays to determine the inter-assay variation (V_1). The inter-assay variation was also determined for dilutions of the standard serum preparation used in 10 assays, where:

$$V_1 = \frac{s}{\bar{x}} \times 100$$

The intra-assay variation (V) was calculated for each dilution of the above specimens, and also each dilution of the serum standard.

iv)   Impression cytology technique.

1.   Principle of the assay.

The effect of vitamin A deficiency on conjunctival morphology is well documented, with goblet cells disappearing early in a deficiency state, whereas epithelial cells enlarge and flatten (Hachell & Sommer, 1984).

Impression cytology is a relatively new technique in which an absorbant filter paper is applied to the conjunctival surface of the eye in order to gain a sample of epithelial and goblet cells. The specimen obtained is examined for epithelial cell size and the presence or absence of goblet cells (Amedee-Manesme et al., 1987b).

-116-
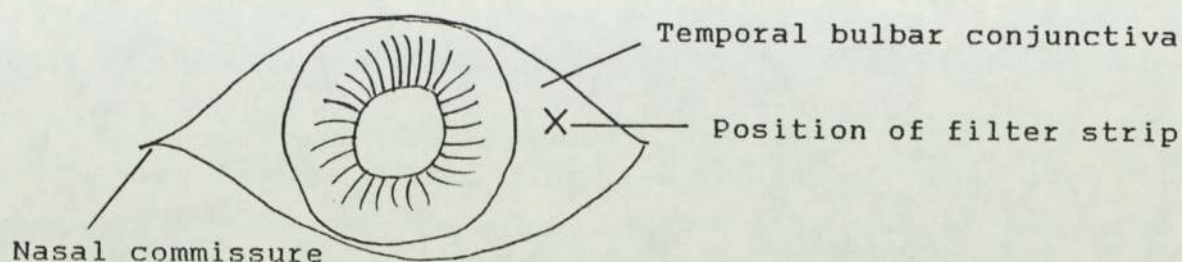
Figure 4.5. shows the position of the filter strip applied to the temporal bulbar conjunctiva to gain a sample of epithelial & goblet cells during the impression cytology technique.

2.   Assay procedure.

a.   Each strip of the millipore filter (pore size 0.22μm) was cut with one irregular edge, so that this end of the filter could be handled without fear of damaging the specimen, which was collected at the opposite end.

b.   The millipore filter strip was applied to the temporal bulbar conjunctiva (Figure 4.5) with gentle finger pressure for 3 seconds. The strip was then removed with a lifting motion, avoiding shearing and twisting. It was then covered with absolute alcohol for at least 10 minutes.

c.   Specimens may be stained immediately or stored at $4^{o}C$ in absolute alcohol, in a sealed container. All specimens should be handled with care, using toothless forceps.

-117-

d. Each strip of filter was hydrated in tap water (5 minutes) before oxidation in 1% periodic acid (5 minutes). The strip was then rinsed in tap water.

e. The strip was then stained with Schiff's reagent for 10 minutes, and developed in a water bath, with frequent changes of water in order to produce a pink colour, followed by a haematoxylin counterstain (1 minute).

f. The strip was again rinsed in a water bath before decolouring in acid alcohol.

g. A blue colour was developed in a saturated solution of Lithium Carbonate, before rinsing in a water bath.

h. To dehydrate and mount the specimen, the strip was placed in 2 successive baths of methanol (3-4 minutes each), followed by 2 successive baths of absolute alcohol (3-4 minutes each), and then 2 final xylene baths (3-4 minutes each).

i. Each specimen was then permanently mounted on a glass slide.

j. Specimens were examined for the presence of goblet cells and the size of epithelial cells under the microscope.

PART III : RESULTS AND DISCUSSION.

CHAPTER 5 : VALIDATION OF COLORIMETRIC ASSAY FOR SERUM

VITAMIN A LEVELS.

i)   Intra-assay variation.

The   intra-assay   variation   for   serum   vitamin   A
concentration was calculated as 3%. However, following
storage   at 4°C for 21 days this had increased   to   8%.   In
contrast,   the   intra-assay   variation   for   carotenoid
concentration (7%) was not affected by storage.   Degradation
of   vitamin   A to products exhibiting absorbance   at   620nm,
probably   caused   the   increased   variation   in   vitamin   A
concentration following storage. Moreover, evaporation might
have   occurred at varying rates,   and to   differing   degrees
resulting in a change in vitamin A concentration.

Specimens   (n=4) were assayed by both the   colorimetric
assay   and by an HPLC method (Thurnham et   al.,   1988).   The
intra-assay   variation was calculated for each assay,   as 2%
for vitamin A,   and as 5% and 4% respectively for carotenoid
concentration.   The   assays   would therefore   appear   to   be
comparable.

ii)   Assay Calibration.

1.   Carotene Calibration

The carotene calibration curve obtained in the   present
study has been shown in **Figure** 5.1.   The factor required for
the   conversion   of absorbance at 450nm to serum   carotenoid
concentration (F_C) was calculated as 18 where:

$$F_C = \frac{1}{slope}$$

-121-
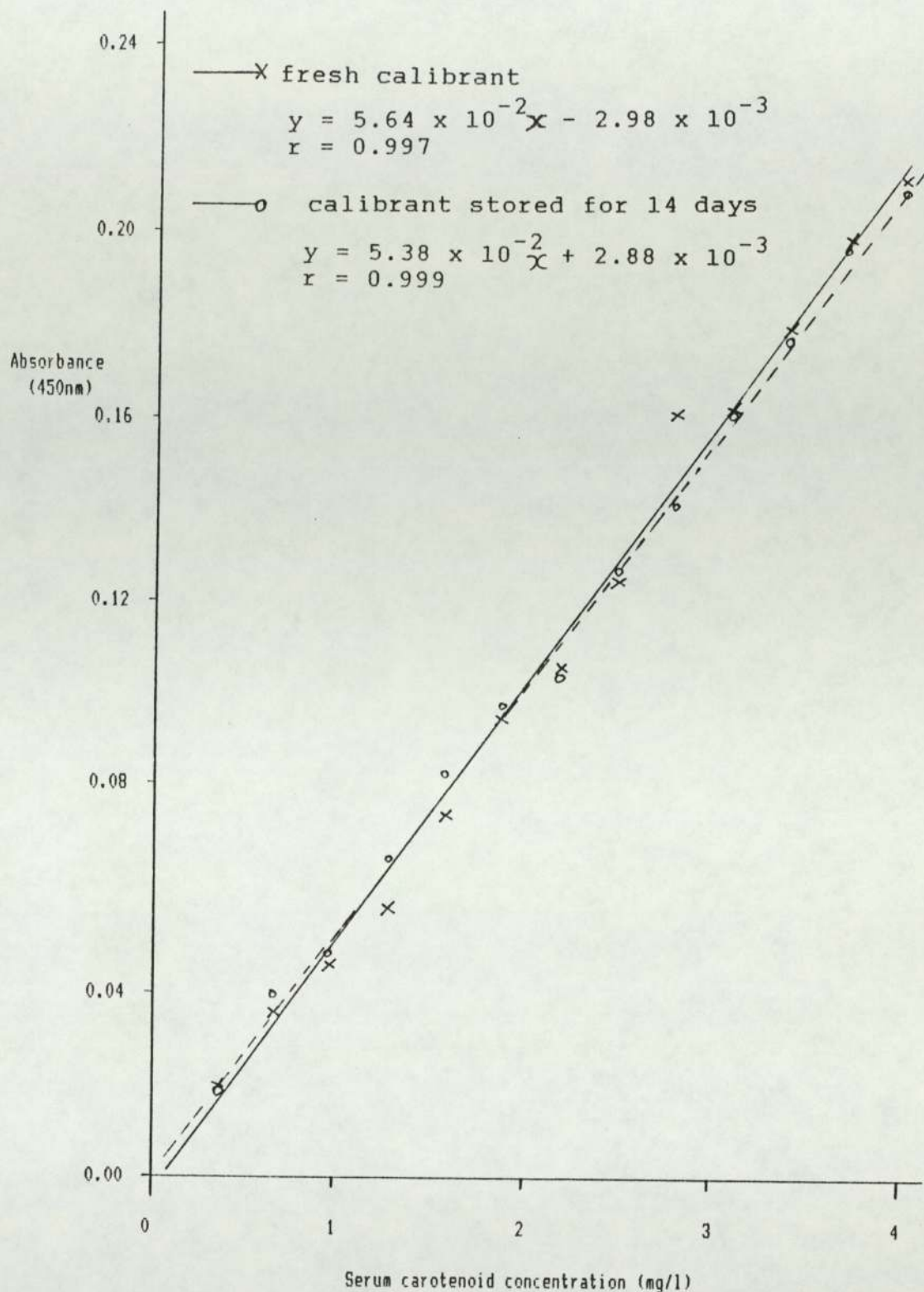
Figure 5.1. represents a standard calibration curve for serum carotenoid concentration, using a solution of beta-carotene in petroleum ether as a model for serum (0.30-4.20mg/l). The curves obtained from fresh calibrant (———X) and following storage of calibrant at 4°C for 14 days (———o) have been shown. Correlation coefficient for fresh calibrant (r) = 0.997, slope = 5.64 x 10^{-2} (n=14). Following storage r = 0.999, slope = 5.38 x 10^{-2}, (n=14).

This factor correlated well with the previous factor of 15 which was used at The Birmingham & Midland Eye Hospital. Storage of the stock carotene solution at $4^{o}C$ for 14 days did not affect carotene concentration.

2.   Carotene Correction.

A correction for carotene was required when determining vitamin A, since carotenoids also exhibit absorbance at 620nm (Frolik & Olson, 1984). The absorbance of serum carotenoid equivalent to each dilution of the carotene solution at 620nm (see chapter 4.i.5) has been shown in Figure 5.2. A correction factor (F_{CC}) of 120 for carotenoid absorbance was calculated from the correction curve, where:

$$F_{CC} = \frac{1}{slope}$$

This factor again correlated well with the previous correction factor (119), considering the high variability of the points on both determinations. The correction factor was also calculated using the carotene recovery data since high variability in the above curve had been noted. The absorbance owing to the carotene solution alone was determined as follows:

$$A_{620nm\ car} = A_{total} - A_{pool}$$
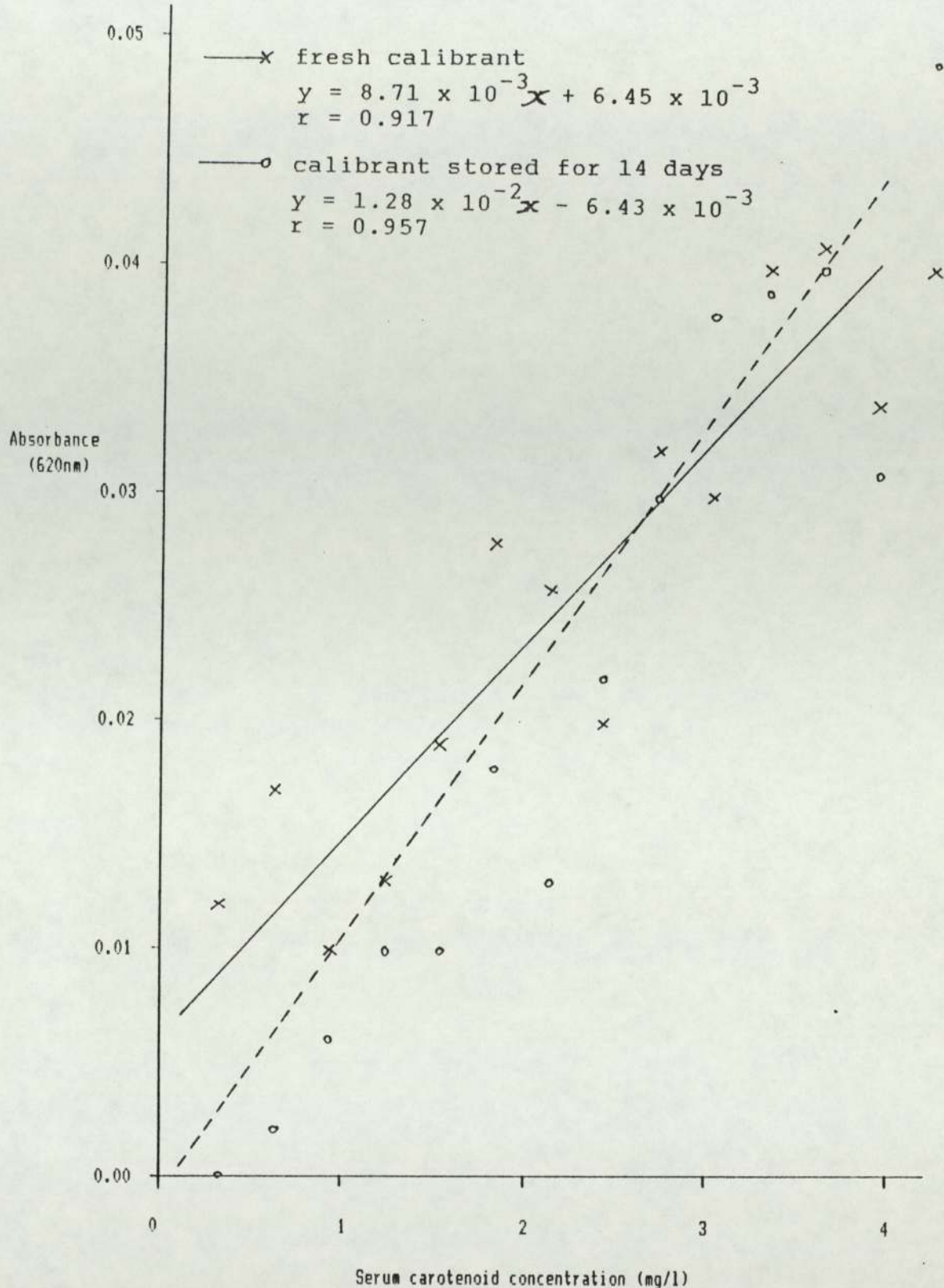
Figure 5.2. represents a standard carotenoid correction
curve for determination of serum vitamin A. The absorbance
at 620nm of solutions of beta-carotene in petroleum
ether, equivalent to a serum concentration of 0.30-4.20mg/1
was measured. The curves obtained from fresh calibrant
(———X) and following storage at 4°C for 14 days (———o)
have been shown. Correlation coefficient (r) of fresh
calibrant = 0.917, slope = 8.71 x 10^{-3}, (n=14). Following
storage r = 0.957, slope = 1.28 x 10^{-2}, (n=12).

The carotene correction curve has been shown in Figure 5.3. The correction factor was calculated as 95, slightly lower than the factor obtained from Figure 5.2. The results remained highly variable, particularly following storage. This may have been due to a relatively high environmental temperature at the time of the experiment, resulting in evaporation.

Since the regression coefficient (r) for the curve in Figure 5.2 was calculated as 0.917, compared with r = 0.869 for Figure 5.3, the factor obtained from Figure 5.2 was employed in the calculation of the serum vitamin A levels.

3.   Carotene recovery.

The mean percentage recovery of carotene, $94\pm1\%$ (n=13) from each dilution of the stock solution, (range 87 to 99%), did not increase following storage of the stock solution for 14 days.

4.   Vitamin A calibration.

The vitamin A calibration curve shown in Figure 5.4 was obtained by plotting the equivalent serum vitamin A concentration for each dilution of the vitamin A (retinol) solution (mean of 2 readings), against absorbance at 620nm. The factor required for the conversion of absorbance at 620nm to serum vitamin A concentration ($F_{vit\ A}$) was calculated from Figure 5.4, as 1.20×10^4, where:
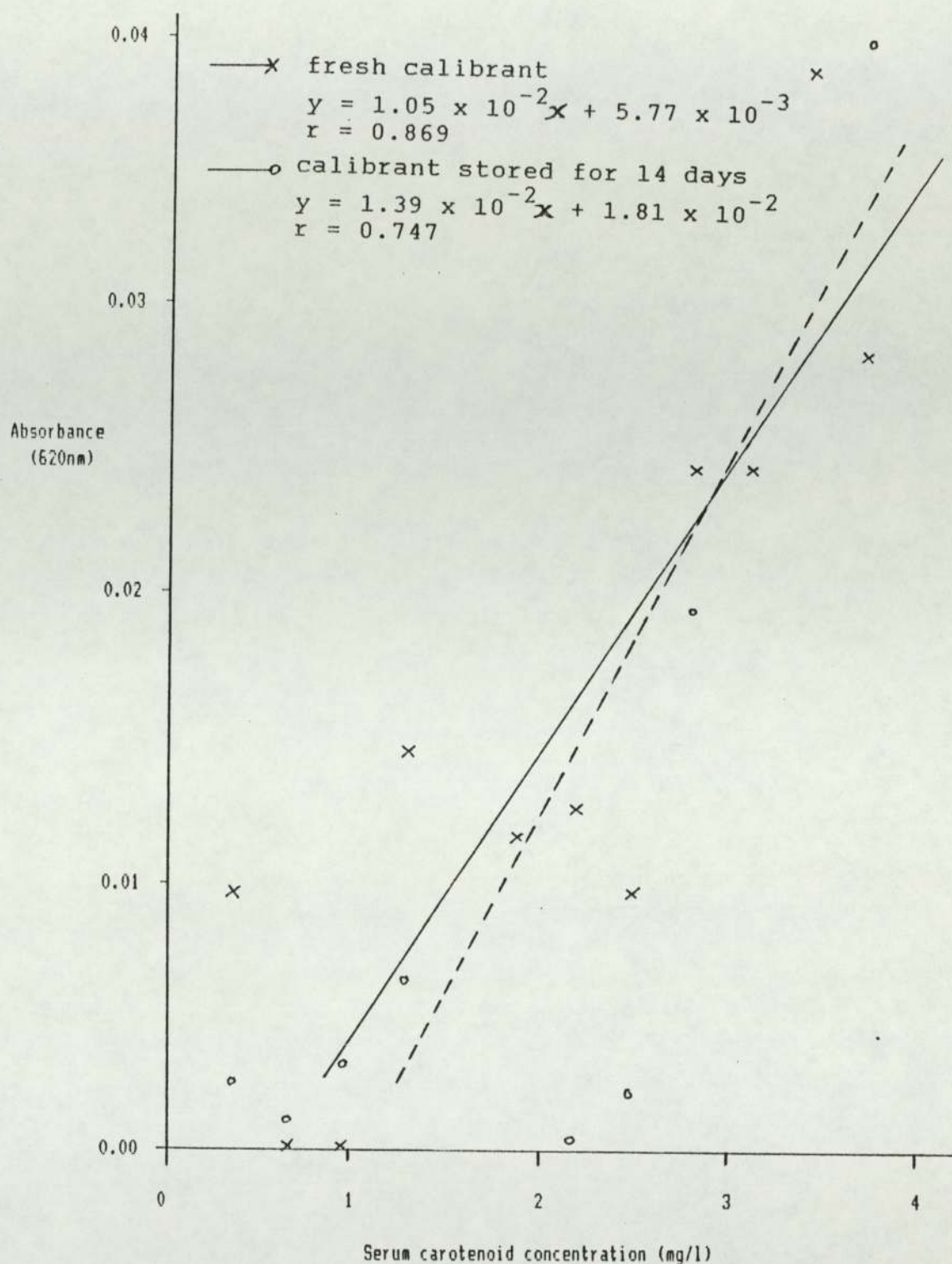
Figure 5.3. represents a standard carotene correction curve
for determination of serum vitamin A, using absorbance at
620nm of beta-carotene recovered from serum. The curves
obtained from fresh serum (————✗) and following storage at
$4^{\circ}C$ for 14 days (————o) have been shown. Correlation
coefficient (r) for fresh serum = 0.869, slope =
1.05×10^{-2}, (n=10). Following storage r = 0.747,
slope = 1.39×10^{-2}, (n=8).

0.50 -

⟶⤬ fresh calibrant

$Y = 8 \times 10^{-5} x + 1.09 \times 10^{-2}$
$r = 0.999$

Absorbance
(620nm)

0.40 -

0.30 -

0.20 -

0.10 -

0.00 -

0   1000   2000   3000   4000   5000

Serum vitamin A concentration (iu/l)
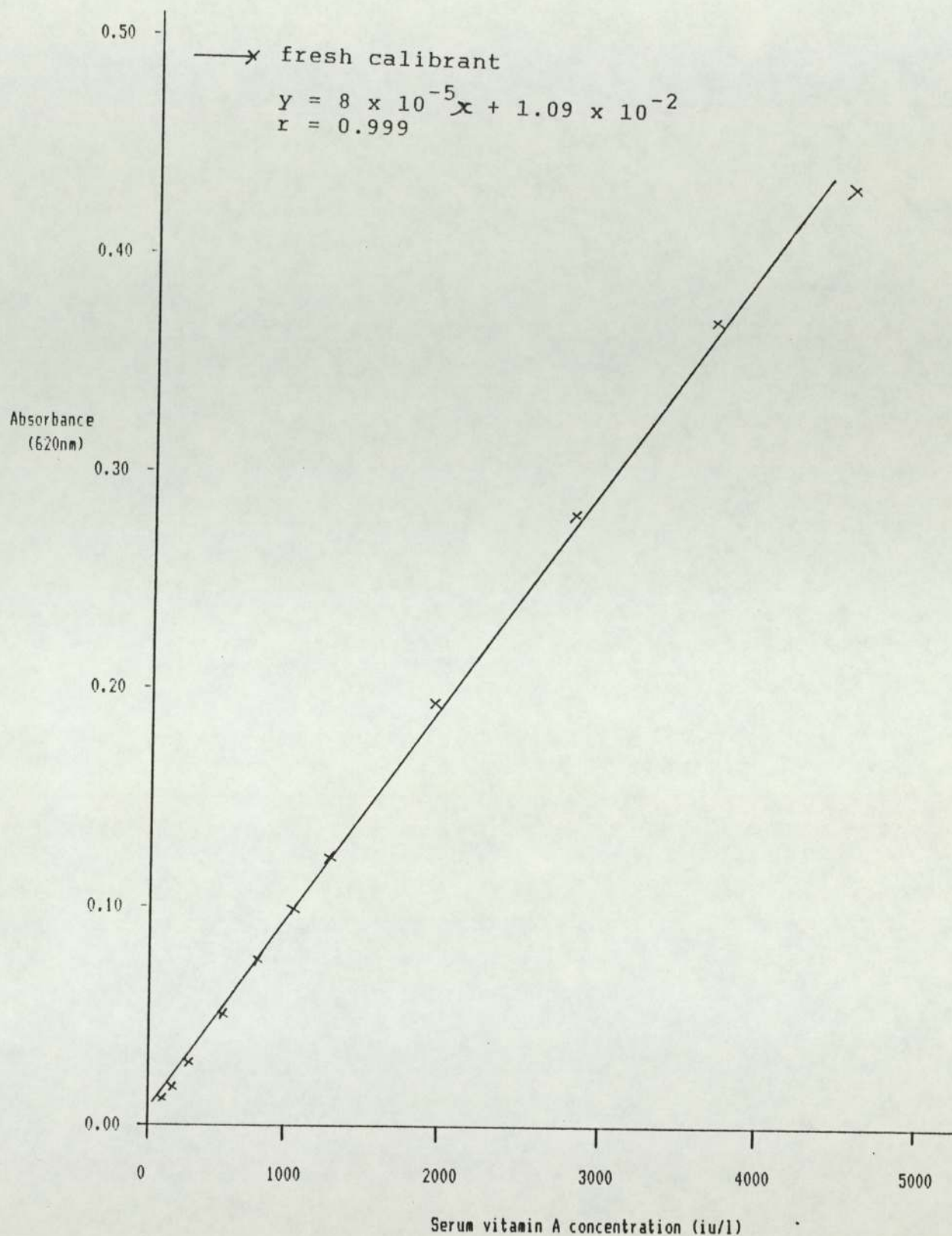
Figure 5.4. represents a standard calibration curve for serum vitamin A concentration, using a solution of vitamin A in chloroform as a model for serum (100-5,000iu/l). Correlation coefficient (r) = 0.999, slope = 8.10^{-5}, (n=11). Each point represents the mean of 2 readings.

$$F_{\text{vit A}} = \frac{1}{\text{slope}}$$

The factor obtained was comparable with the previous factor 1.19×10^4 determined by Cowen (unpublished data). The dilutions of vitamin A solution in chloroform did not result in any absorbance at 450nm since no carotene was present, confirming that vitamin A did not interefer with carotenoid determination.

5. Calculation of serum vitamin A and carotenoid levels.

The factors obtained from the calibration curves (see above) were incorporated into the following equation to allow rapid calculation of serum vitamin A and carotenoid levels:

$$[\text{Car}]_S = A_{450\text{nm}} \times 18$$
$$(\text{mg/l})$$

$$[\text{Vit A}]_S = (A_{620\text{nm}} \times 1.2 \times 10^4) - (A_{450\text{nm}} \times 18 \times 120)$$
$$(\text{iu/l})$$

iii) Investigation of variables associated with the assay.

1.   Haemolysis.

The effect of haemolysis on a serum pool with a mean vitamin A concentration of 1.94µmol/l (n=2), and a mean carotenoid concentration of 1.14 mg/l (n=2) prior to addition of the haemolysate was investigated. The haemolysate contained no vitamin A but carotenoid content was 0.42 mg/l (n=2), however since the volumes of the haemolysate added to the pool were so small (1.66-10µl), the effect on vitamin A and carotenoid concentration was unlikely to be important. The effect of haemolysis on serum vitamin A and carotenoid concentrations has been shown in Figure 5.5.

The degree of haemolysis ranged from severe (10µl of haemolysate produced a deep red colouration of the serum) to mild (1.66µl of haemolysate produced a slightly pink colouration) however, there was no significant change in vitamin A or carotenoid concentration in the presence of the increasing degrees of haemolysis.

2.   Duration of vortex mixing.

The effect of the duration of vortex mixing on the mean vitamin A and carotenoid concentrations of a serum pool has been shown in Figure 5.6. Extraction of vitamin A and carotenoid into the petroleum ether appeared to be complete

-129-

a.
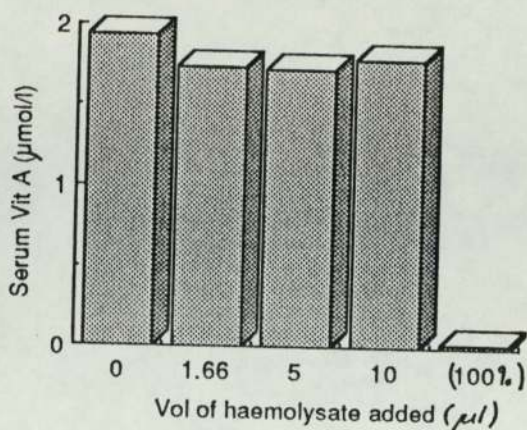
b.
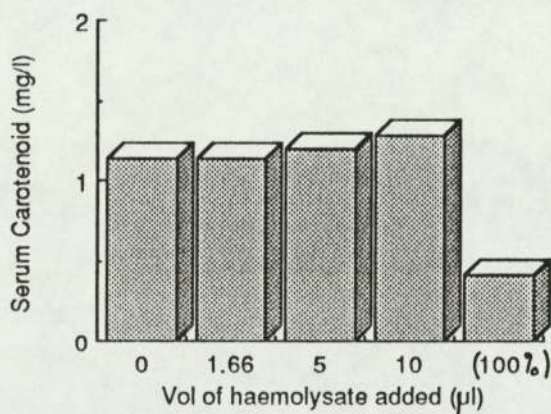
Figure 5.5. Histograms representing the effect of haemolysis
on serum vitamin A (a.) and carotenoid (b.) concentration.
The abscissa show volume of haemolysate (1.66-10μl) added to
1ml of a serum pool, and the ordinate shows serum
concentration of vitamin A (μmol/1) (a.) or carotenoid
(mg/1) (b.). The histobars represent the mean serum
concentration (n=2), and the serum concentrations of the
haemolysate itself have been included for comparison (100%).
Haemolysis did not affect serum concentration of vitamin A
or carotenoid.

following vortex mixing for 1 min, since a significant increase in vitamin A and carotenoid concentration, was found following vortex mixing for 1 min (p<0.001). There was no further change in vitamin A concentrations as the time of vortex mixing was increased from 1 to 5 mins.

3.   Specimen volume.

Serum vitamin A and carotenoid levels of aliquots (of varying volume) taken from a serum pool with a relatively high vitamin A content (2.50μmol/l) (n=2) but normal carotenoid concentration (1.02 mg/l) (n=2) have been shown in **Figure 5.7** . There was a trend for the concentration of vitamin A to increase as specimen volume was decreased, however this was only significant for volumes of 0.2 and 0.3ml (p<0.05). Likewise, a similar trend was noted in carotenoid concentration, the increase in carotenoid concentration being significant for all volumes when compared with the 1.0ml aliquots (p<0.001 or p<0.05). Increased variability in the results was noted in all volumes < 1.0ml.

4.   Short term storage of serum.

There was no significant difference in the vitamin A concentration of the aliquots of serum stored under different conditions for 5 days, except a fall in vitamin A concentration when aliquots were stored at $15-18^{\circ}$C, unprotected from light, for periods longer than 3 days.
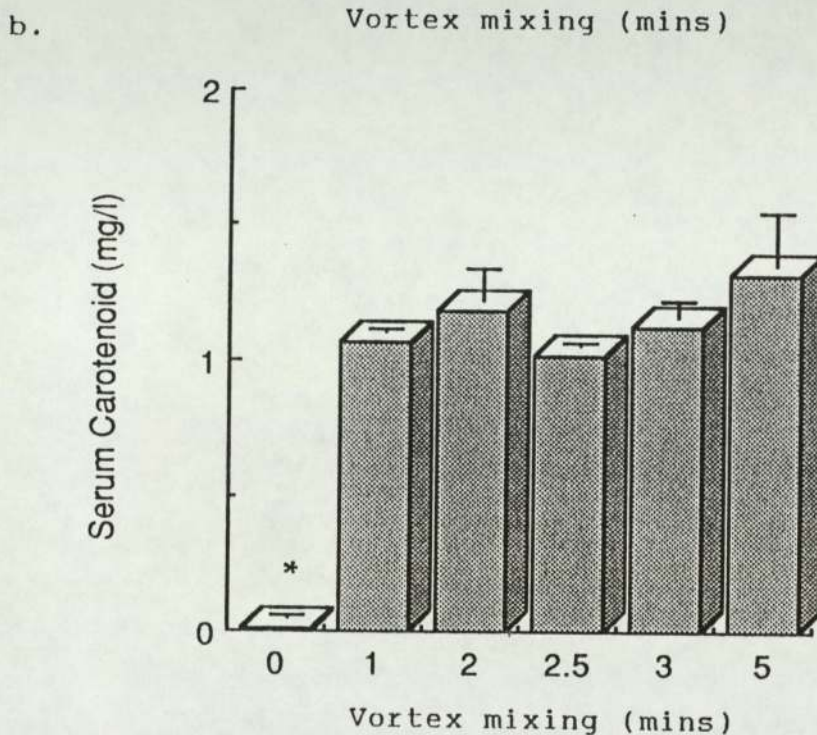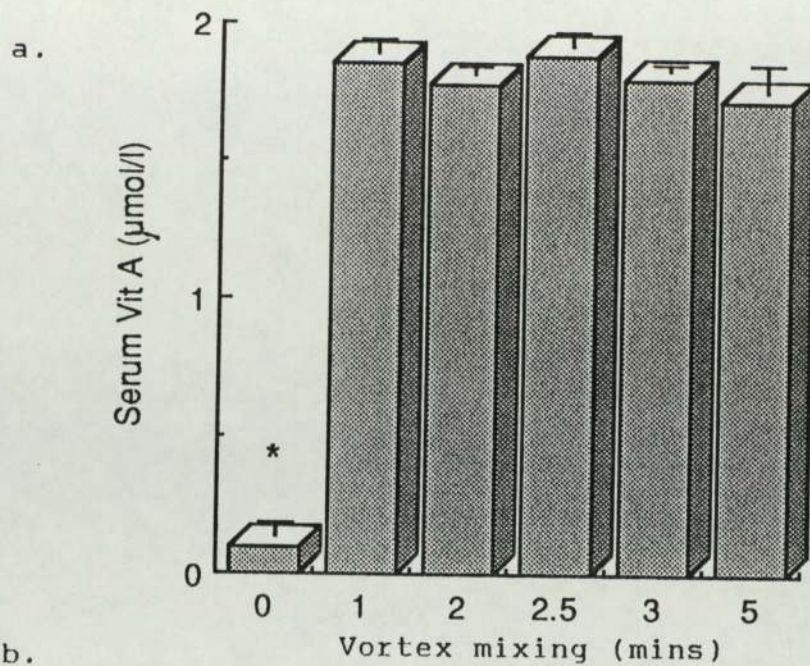
Figure 5.6. Histograms representing the effect of duration of vortex mixing on serum concentration of vitamin A (a.) and carotenoid (b.). The abscissa show duration of vortex mixing (mins), whilst the ordinate show serum concentration of vitamin A (µmol/l) (a.) or carotenoid (mg/l)(b.). Histobars represent the mean serum concentration (n=3), with the central vertical bar giving the SEM. Extraction of vitamin A and carotenoid appeared to be complete following vortex mixing for 1 min (p<0.001), since no further increase in concentration occurred

a.
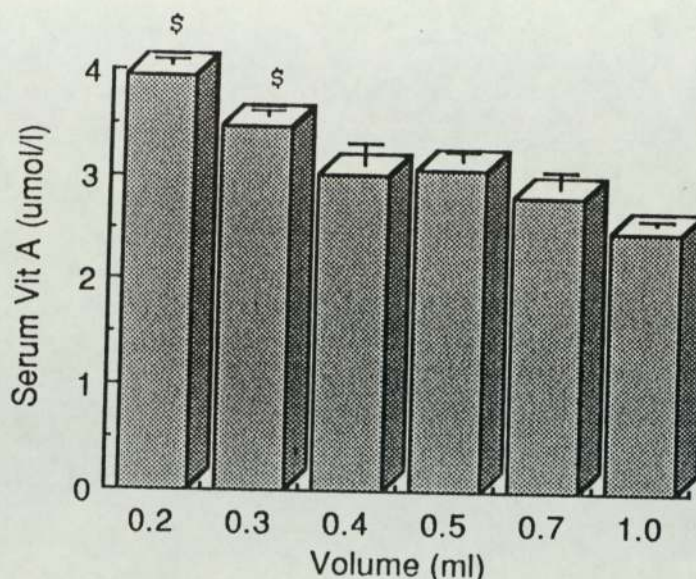
b.
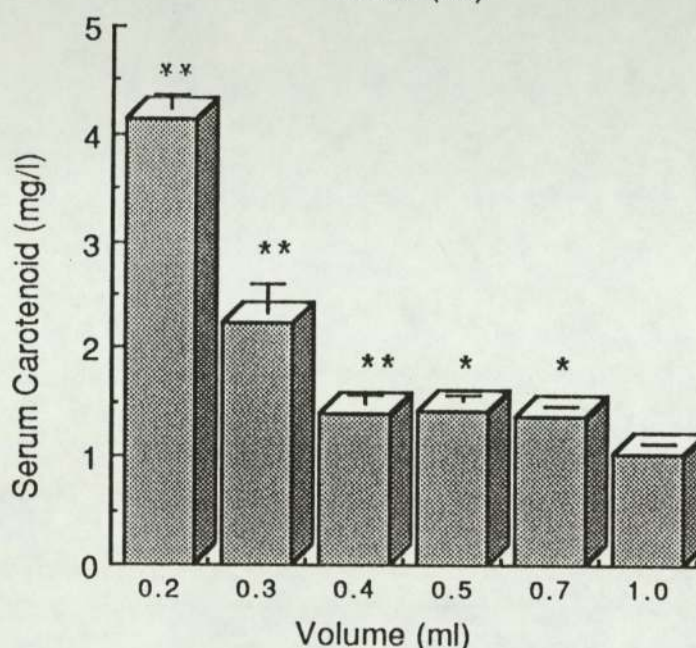

```
*      p < 0.001
**     p < 0.005
$      p < 0.05
```

Figure 5.7. representing the effect of specimen volume on serum concentration of vitamin A (a.) and carotenoid (b.). The abscissa show volume of serum (ml), whilst the ordinate shows serum concentration of vitamin A (µmol/l) (a.) or carotenoid (mg/l) (b.). Histobars represent mean serum concentration (n=3), except for 1.0ml specimens where n=2 and central vertical bars give SEM. Serum vitamin A concentration was significantly higher in specimens of 0.2ml or 0.3ml (p<0.05). Conversely, serum carotenoid concentration was increased in all volumes < 0.7ml (p<0.001 or p<0.005).

Furthermore, the aliquots stored for 4, but not 5 days at 15-18°C, which were protected from light also showed a significant decline in vitamin A content (p<0.05). These findings have been summarised in **Figure 5.8.**

The decline in vitamin A content following storage for 4 days was less (p<0.05) when the aliquots were protected from light.

There was no significant change in carotenoid concentration of the serum pool, except an increase in aliquots stored at 4°C or 15-18°C (unprotected from light) for 5 days (p<0.05). The mean carotenoid concentration of the aliquots has been given in **Figure 5.9.**

These results indicated that storage at 4°C was not ideal for carotenoid analysis, even in the short term. This phenomenon requires further investigation.

5. Long term storage of serum.

Aliquots of 5 to 9 specimens were stored at 4°C and -20°C for periods ranging from 10 to 84 days, prior to analysis. The serum vitamin A and carotenoid concentrations of these specimens have been shown in **Figures 5.10 and 5.11.**

A significant increase in apparent vitamin A content was noted after storage at 4°C for 10 days (p<0.05) and 78 days (p<0.05), when compared with the fresh specimens. Moreover, the colour yield of specimens stored at -20°C for

-134-
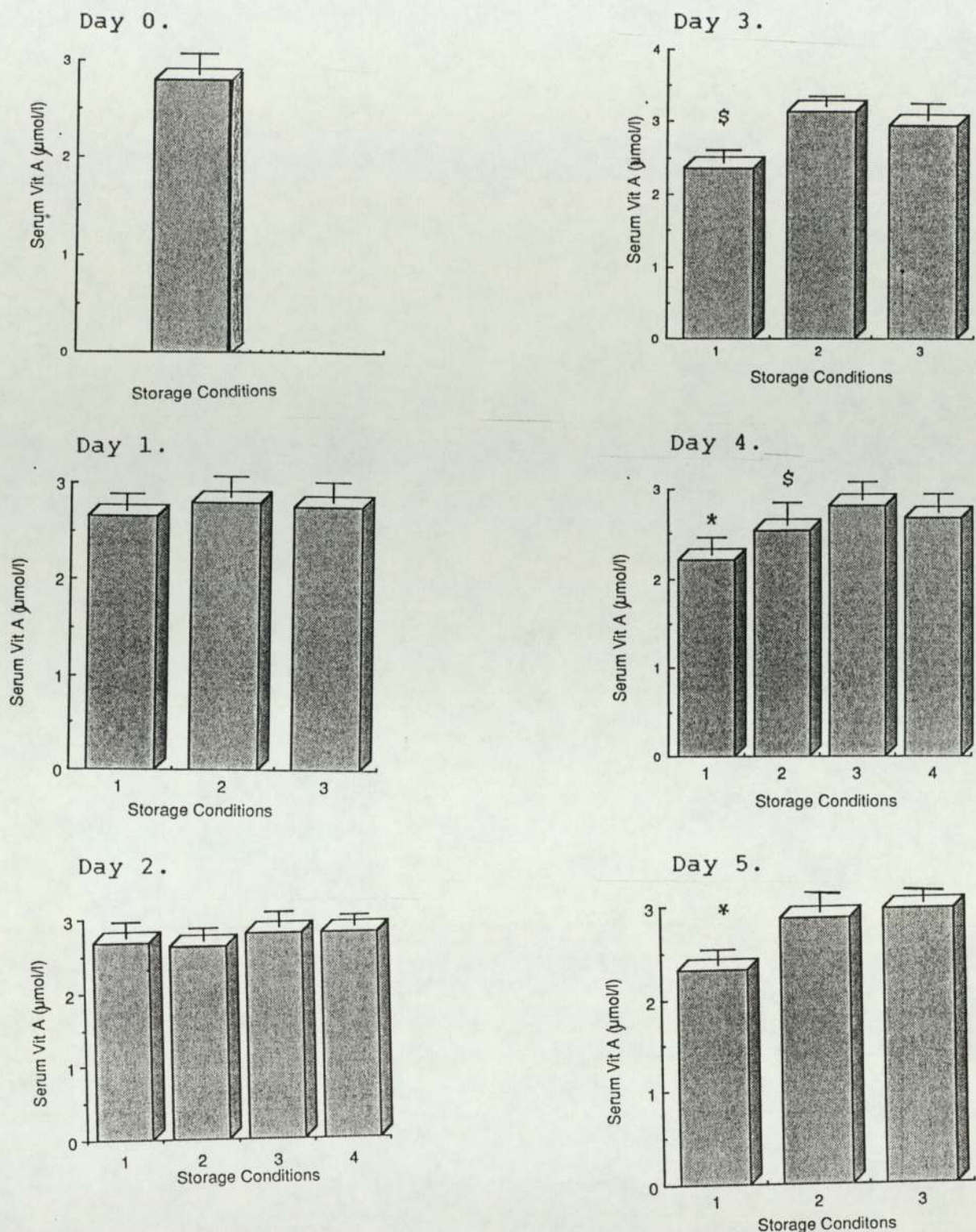
Figure 5.8. Histograms representing the effect of storage of specimens for 1-5 days on serum vitamin A concentration. The abscissa show storage temperature of $15-18^{o}$C (1), $15-18^{o}$C protected from light (2), 4°C (3) and -20°C (4). The ordinate shows serum vitamin A concentration (μmol/l), and the histobars represent mean serum concentration (n=6) with central vertical bars giving the SEM. Storage of specimens for 1-5 days only affected serum vitamin A concentration when specimens were stored at $15-18^{o}$C unprotected form light for > 3 days (p<0.05 or p<0.001). Serum vitamin A concentration also increased in specimens stored at $15-18^{o}$C and protected from light for 4 but not 5 days (p<0.05).
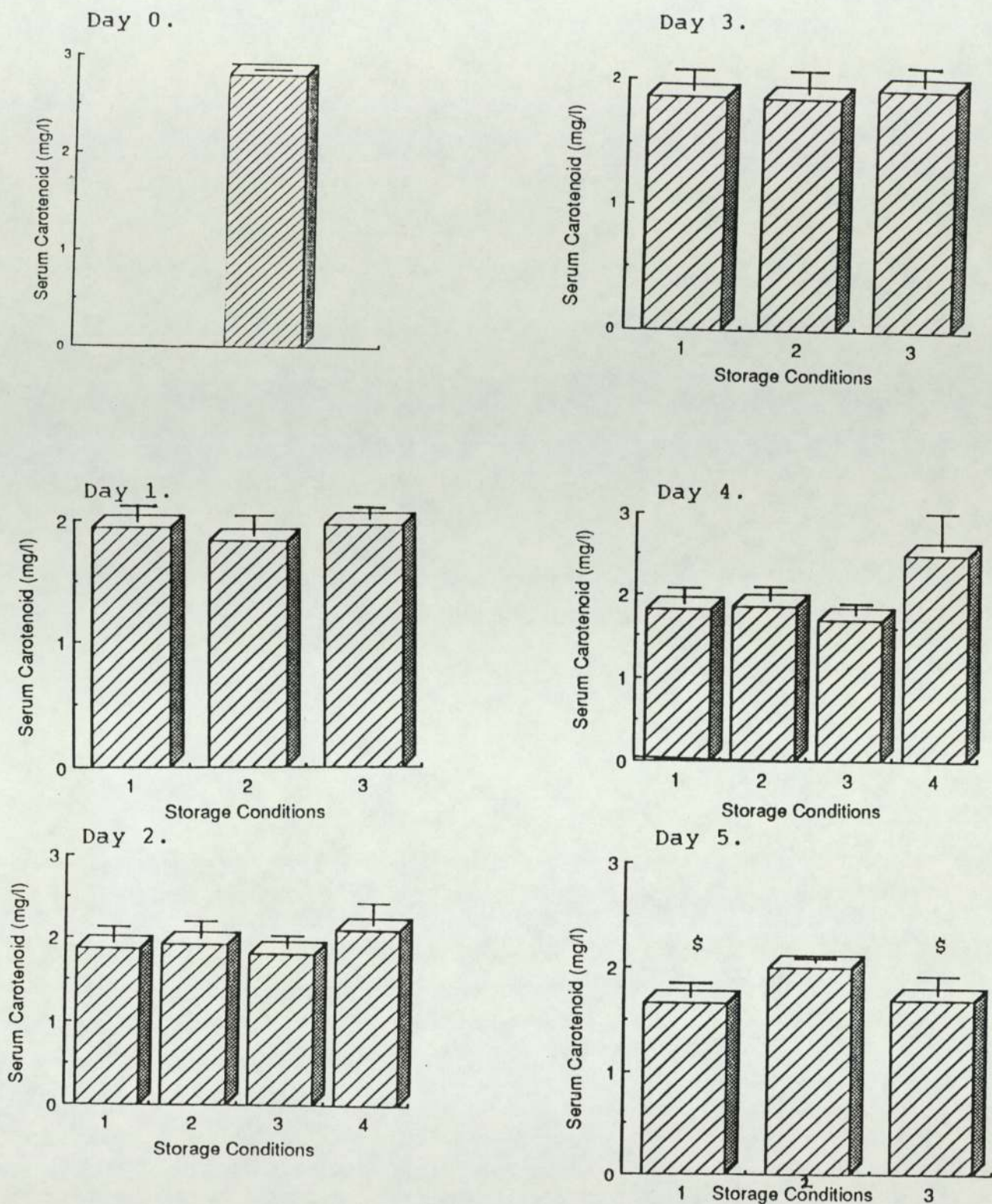
$ p < 0.05

Figure 5.9. Histograms representing the effect of storage of specimens for 1-5 days on serum carotenoid concentration. The abscissa show storage temperature of 15-18°C (1), 15-18°C protected from light (2), 4°C (3) and -20°C (4). The ordinate shows serum carotenoid concentration (mg/1), and histobars represent mean serum concentration (n=6) with central vertical bars giving the SEM. Storage of specimens for 5 days at 15-18°C unprotected from light or 4°C resulted in a significant decrease in serum carotenoid concentration (p<0.05).

longer than 10 days was noted to be purple rather than the expected deep blue. Whilst this did not significantly affect vitamin A content, interpretation of the assay was more difficult, since the absorbance must be determined prior to formation of the purple colouration in order to avoid overestimation. In agreement with the present study Parkinson and Gal (1972) noted increased colour yields following storage at -20°C, for one week.

The purple colouration was also noted in specimens stored at 4°C, for 78 days. By 84 days the formation of the purple colouration was more rapid, and in one specimen accurate determination of vitamin A was not possible. Bacterial growth was also noted in specimens stored for 84 days. The percentage error produced by reading the absorbance of the purple rather than the deep blue colouration was determined by reading the peak absorbance of the blue, and then the purple colouration in 6 specimens which had been stored for 84 days. The mean increase in absorbance of $11\pm2\%$ (n=6) (range 5 to 20%) corresponded to a mean increase of $14\pm4\%$ (n=6) in vitamin A content (range 5 to 31%).

A significant decrease in carotenoid concentration was noted in specimens stored at 4°C for 56 ($p<0.05$), 78 ($p<0.001$), and 84 days ($p<0.05$), indicating that prolonged storage also affects carotenoid concentration, however this effect of storage was probably less variable than that on
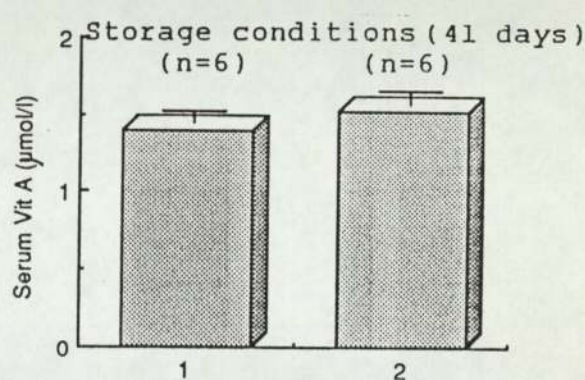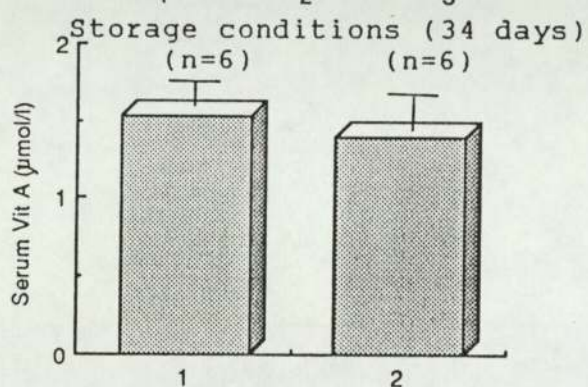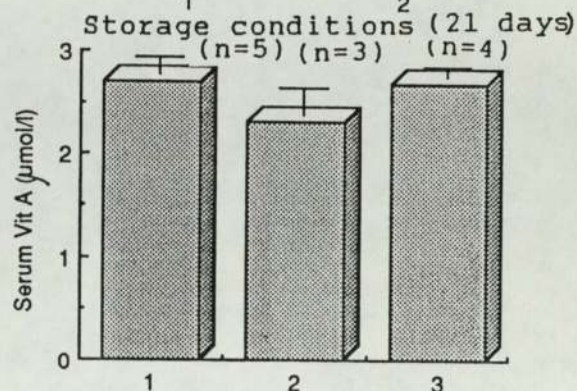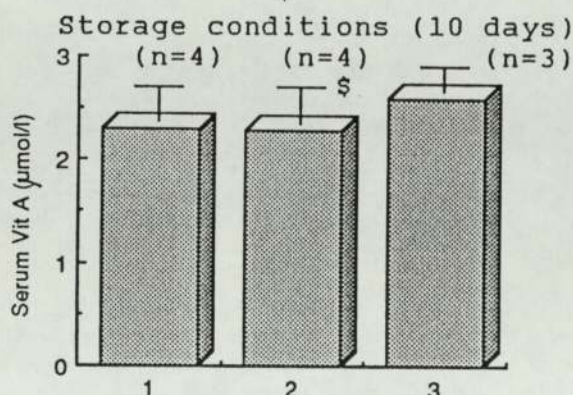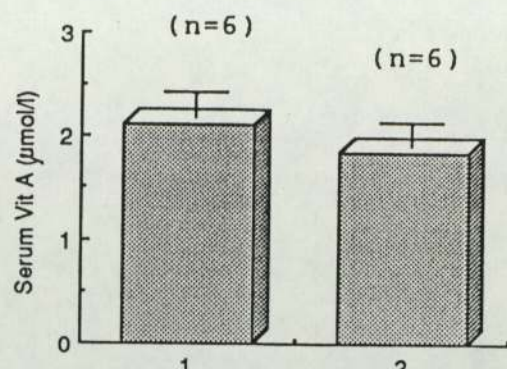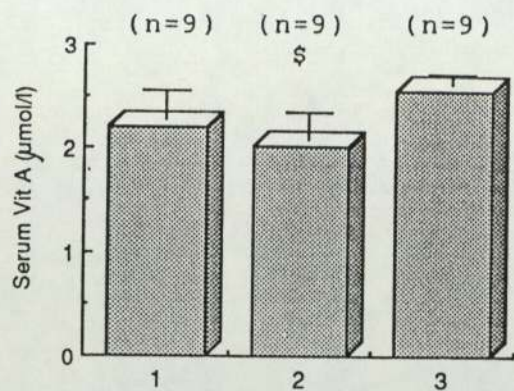
-137-
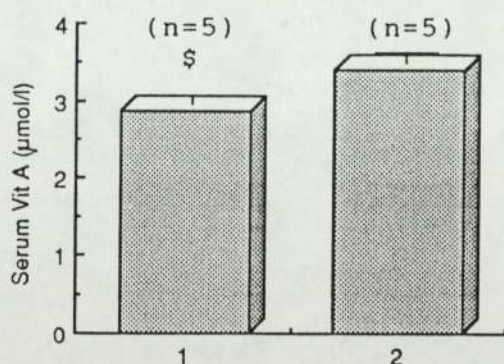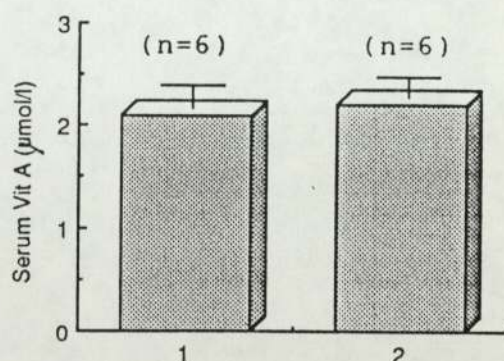
$ p < 0.05

Figure 5.10. Histograms representing the effect of storage of specimens for 10-84 days on serum vitamin A concentration. The abscissa show fresh specimens (1) or storage temperatures of $-4^{\circ}C$ (2) or $-20^{\circ}C$ (3), whilst the ordinate shows serum vitamin A concentration (μmol/l). Histobars represent mean serum concentration with central vertical bars giving the SEM. The number of specimens has been given in parenthesis. A significant increase in serum vitamin A concentration was noted following storage at $-4^{\circ}C$ for 10 and 78 days ($p<0.05$).
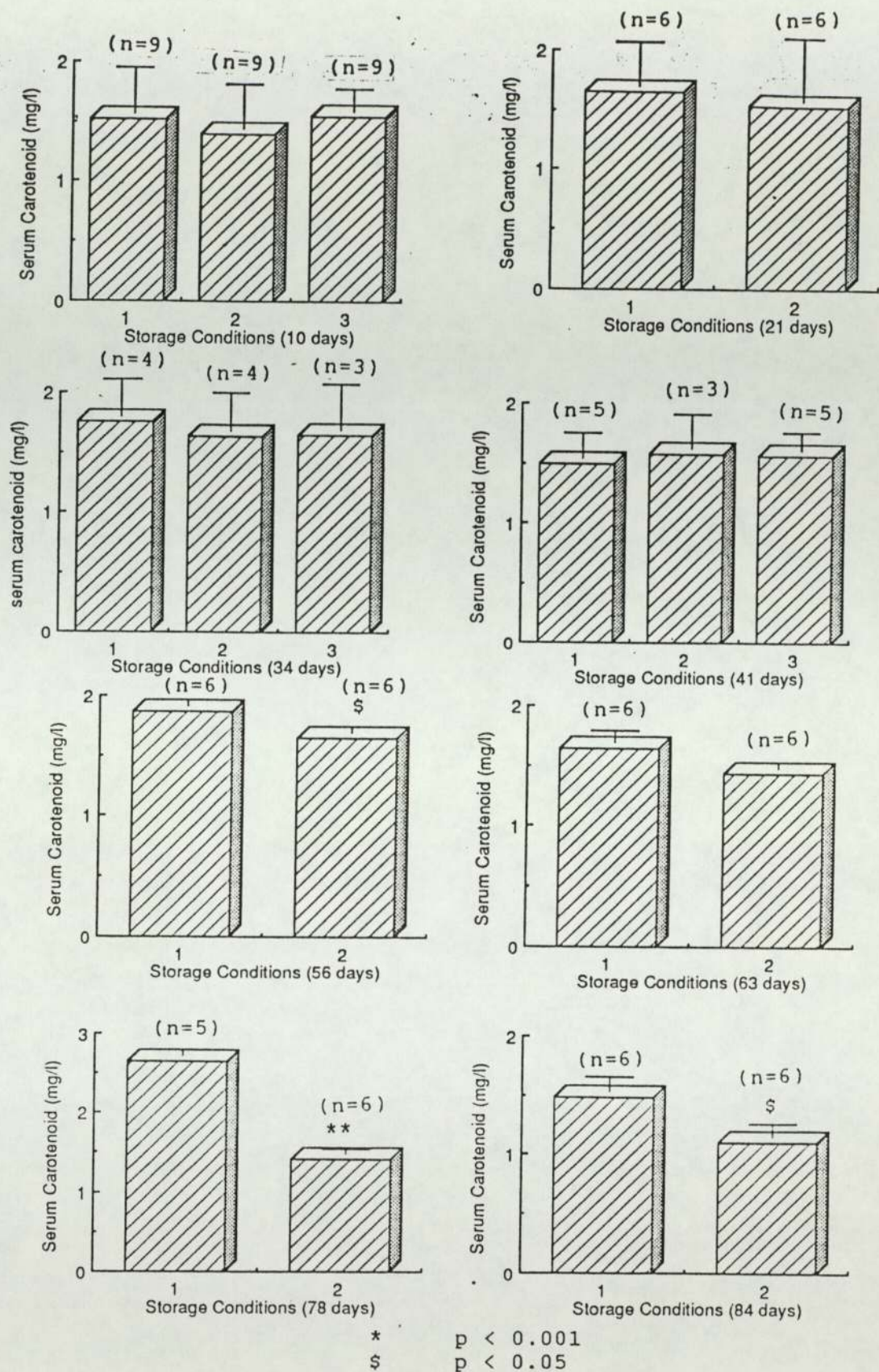
Figure 5.11. Histograms representing the effect of storage
of specimens for 10-84 days on serum carotenoid
concentration. The abscissa show fresh specimens (1) or
storage temperature of $-4^{\circ}C$ (2) or $-20^{\circ}C$ (3), whilst the
ordinate shows serum carotenoid concentration (mg/l).
Histobars represent mean serum concentration, with central
vertical bars giving the SEM. The number of specimens has
been given in parenthesis. Storage of specimens at $-4^{\circ}C$ for
56, 78 and 84 days resulting in a significant decrease in
serum carotenoid concentration ($p<0.05$ or $p<0.001$).

-139-

vitamin A content. The limited data on storage at -20°C suggested that carotenoid was more stable when serum was stored at the lower temperature.

iv) Discussion of variables associated with the assay.

1. Haemolysis.

Haemolysis was noted in a number of specimens in the present study. However, even when a severe degree of haemolysis producing a deep red colouration of the serum was induced, the vitamin A and carotenoid concentration of specimens was unaffected; despite previous suggestions that haemolysis might affect the apparent vitamin A and carotenoid concentration, (Frolik & Olson, 1984).

2. Duration of vortex mixing.

The extraction of vitamin A and carotenoid from serum appeared to be complete following vortex mixing for 1 min since further mixing did not affect serum concentration. Loss of vitamin A or carotenoid concentration might result from evaporation, even from well stoppered tubes if vortex mixing time was prolonged. Therefore mixing time should be limited to the minimum (1 min) required for complete extraction of vitamin A and carotenoids.

3. Specimen volume.

Since specimens were frequently obtained from young children with poor venous access, 1ml of serum was seldom

available for analysis, however Cowen (unpublished data) had noted that serum vitamin A and carotenoid levels from such small specimens were higher than those of a larger aliquot from the same source. In the present study, the apparent concentrations of both vitamin A and carotenoid appeared to increase as the specimen volume was decreased. Whereas this increase was only significant in volumes of 0.3 and 0.2ml for vitamin A, the increase in carotenoid concentration was significant for volumes of 0.7ml and less. Fortunately, a correction factor was applied to the vitamin A determination in order to correct for any interfering carotenoid. The results indicated that determination of carotenoid concentration on small specimens was not accurate, however vitamin A concentration could be determined on specimens of 0.4ml or more.

4.   Short term storage.

The effects of storage were investigated since concerns over the instability of vitamin A and carotenoids in serum have been well documented (Frolik & Olson, 1984). The results of the present study indicated that over a 5 day period, storage at 4°C or -20°C was acceptable, but that specimens should probably not be stored at $15-18^{\circ}$C for longer than 2 days. However, this limited stability at room temperature ensured that specimens which were either not collected from the ward immediately, or which were handled in a laboratory for a period of hours, and then posted

-141-

overnight, were unlikely to loose vitamin A potency.
Carotenoids appeared to be less stable following storage,
even for 5 days. Fortunately, a correction was made for
carotenoids in all determinations of vitamin A.

5.   Long term storage.

The effect of long term storage on vitamin A content
did not appear to be directly related to the length of the
storage period. However, analysis should be completed within
one week, to allow for specimens which might degrade more
rapidly than anticipated. Storage at $-20^{o}C$ was not
advisable, since increased colour yields were noted, even
after short periods of storage (7 to 10 days). These
findings were in agreement with those of Parkinson and Gal
(1972), who noticed increased colour yields with the Carr-
Price reaction, following storage at $- 20^{o}C$ for one week
and a 350% increase after 9 months.

Frolik and Olson (1984) suggested that specimens should
be stored at $-20^{o}C$ if analysis is to be delayed, however
Parkinson and Gal (1972) demonstrated increased colour
yields when specimens were stored under such conditions, as
a result of formation of chromophoric compounds, probably 7-
dehydrocholesterol which give a false positive with TFA
(Frolik & Olson, 1984). In contrast, Neald and Pearson
(1963) suggested that purple colouration with TFA resulted
from formation of vitamin A acid (Retinoic acid).

-142-

iv) Conclusions

The results of the present study indicated that the specimen volume should be at least 0.4ml for vitamin A determination, however a reliable measurement of carotenoid concentration could only be completed on specimens > 0.7ml. Although the specimens should be protected from light, vitamin A and carotenoids were sufficiently stable to allow normal handling of specimens. Prolonged storage of specimens could not be recommended since the results of delayed analysis became variable, particularly for carotenoid concentration. The vitamin A and carotenoid concentration were however unaffected by haemolysis, therefore such specimens could be assayed reliably. Moreover, a 1 min duration of vortex mixing appeared to be adequate for the extraction of vitamin A and carotenoid from serum.

On the basis of these findings, any specimen < 0.4ml was excluded from analysis for serum vitamin A in the present study, whilst specimens < 0.7ml were excluded from analysis of serum carotenoid concentration. Since storage appeared to have a variable effect on the apparent serum vitamin A and carotenoid levels, analysis was completed within 7 days.

CHAPTER 6 : SERUM VITAMIN A LEVELS IN MALABSORPTIVE STATES.

i)   Serum vitamin A levels in adult controls.

The healthy adult controls had a mean serum vitamin A
level of $2.15\pm0.16\mu mol/l$ (n=9), which fell within the
published normal ranges ($0.75-2.85\mu mol/l$) (see chapter 11.i)
(Eastham, 1985, Lentner, 1984).

ii) Serum vitamin A levels in liver transplant recipients.

Serum vitamin A levels were available prior to and
following liver transplantation in 5 patients, four of whom
had received vitamin A supplements prior to the transplant.
The serum vitamin A levels obtained have been summarised in
Table 6.1.

Subject		Pre Transplant				Post Transplant		
	(n)	Serum Vit A Mean (SEM) ($\mu mol/l$)	Dose Vit A (iu/day)	(n)	Serum Vit A Mean (SEM) ($\mu mol/l$)	Dose Vit A (iu/day)
1	(2)	0.31	————	(4)	1.57 (0.04)	——[$]
2	(2)	0.14	2,500	(1)	1.34	——[$]
3	(2)	0.59 (0.10)	2,500	(5)	1.70 (0.35)	——[$]
4	(3)	0.95 (0.01)	25,000	(4)	1.60 (0.32)	2,500[$]
5	(7)	1.48 (0.13)	2,500	(5)	0.71 (0.08)	2,500

[$] $p < 0.05$

Table 6.1. Serum vitamin A levels in subjects pre- and post
liver transplantation.

All but one patient demonstrated a significant increase in serum vitamin A levels following liver transplantation (p<0.001), despite discontinuation of vitamin A supplements in three of them, and reduction in supplementation in one. Serum vitamin A levels in the final subject actually fell to a mean of $0.71 \pm 0.08 \mu mol/l$ (n=5) following the liver transplant (p<0.05), despite continuation of vitamin A supplementation.

The serum vitamin A levels obtained from the liver transplant recipients ($1.48 \pm 0.08 \mu mol/l$, n=31) (Figure 6.1) were significantly lower than the adult controls (p<0.05), although the serum vitamin A levels did remain within the normal range for age ($1.00-2.30 \mu mol/l$) (Lentner, 1984). Oral supplementation with 2,500iu ($334 \pm 102 iu/kg$) of vitamin A did not result in a significant increase in serum vitamin A levels in the liver transplant recipients, therefore the supplemented and unsupplemented patients were combined prior to comparison with further groups of patients.

A rapid fall in serum vitamin A levels, from $1.60 \pm 0.16 \mu mol/l$ (n=8) to $0.70 \pm 0.06 \mu mol/l$ (n=4) was observed in subject 4 (Table 6.1) and another subject (not included in above analysis) during periods of acute and chronic rejection (p<0.05). Serum vitamin A levels then rose again following treatment of the rejection or re-transplantation ($1.41 \pm 0.18 \mu mol/l$, n=5) (p<0.05). These serum vitamin A levels were not significantly different from the levels obtained prior to the episodes of rejection.
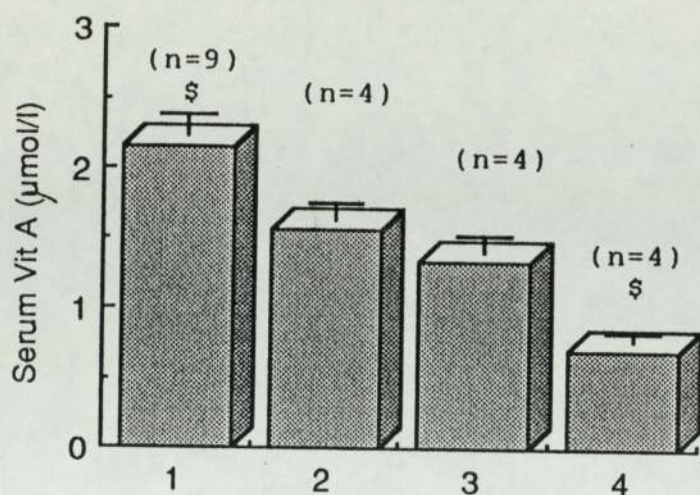
Where:

1   =   Adult controls
2   =   Unsupplemented liver transplant recipients
3   =   Liver transplant recipients receiving 2,500iu
          vitamin A/day
4   =   Liver transplant recipients during periods of
          rejection

$ p < 0.05

Figure 6.1. Histogram representing serum vitamin A levels in liver transplant recipients. The abscissa distinguishes between liver transplant recipients who were receiving vitamin A supplements (2,500iu/day) (3) from those who remained unsupplemented (2), and those in whom rejection of the donar liver was occurring (4). The ordinate shows serum vitamin A level (µmol/l). Histobars represent mean serum level, with the central vertical bar giving the SEM. The number of specimens has been given in parenthesis. Liver transplant recipients were found to have serum vitamin A levels significantly lower than those found in adult controls (p<0.05). Supplementation with 2,500iu of vitamin A did not significantly affect serum vitamin A levels, however episodes of rejection were associated with a fall in serum vitamin A levels (p<0.05).

-147-

iii) Serum vitamin A levels in chronic liver disease.

1.   Prior to vitamin A supplementation.

Specimens (n=52) were obtained from 29 patients (16 male, 11 female) with chronic liver disease (0.50-15 years) who were not receiving vitamin A supplements, and these were compared with 39 specimens from 8 liver transplant recipients (0.83-14 years) (Figure 6.2). The serum vitamin A levels in patients with glycogen storage disease, cholestasis, Wilson's disease and hyperlipidaemia were not significantly lower than those obtained from the liver transplant recipients. In contrast, patients with EHBA, chronic active hepatitis, neonatal hepatitis ($p<0.001$) and liver cysts ($p<0.05$) had significantly lower serum vitamin A levels than the liver transplant recipients.

2.   Following supplementation with 2,500iu of vitamin A.

Specimens (n=117) were also were obtained from 47 patients (25 male, 22 female) with chronic liver disease (0.08-9.00 years), who were receiving an oral daily dose of 2,500iu (347 ± 29iu/kg) of vitamin A (Ketovite Liquid). These patients were compared with 8 liver transplant recipients (0.83-14.00 years), and each group of patients (as defined by diagnosis) was also compared with corresponding groups, who had received no vitamin A supplements where possible (see above). The mean serum vitamin A levels for each group of patients has been summarised in Figure 6.3.

-148-
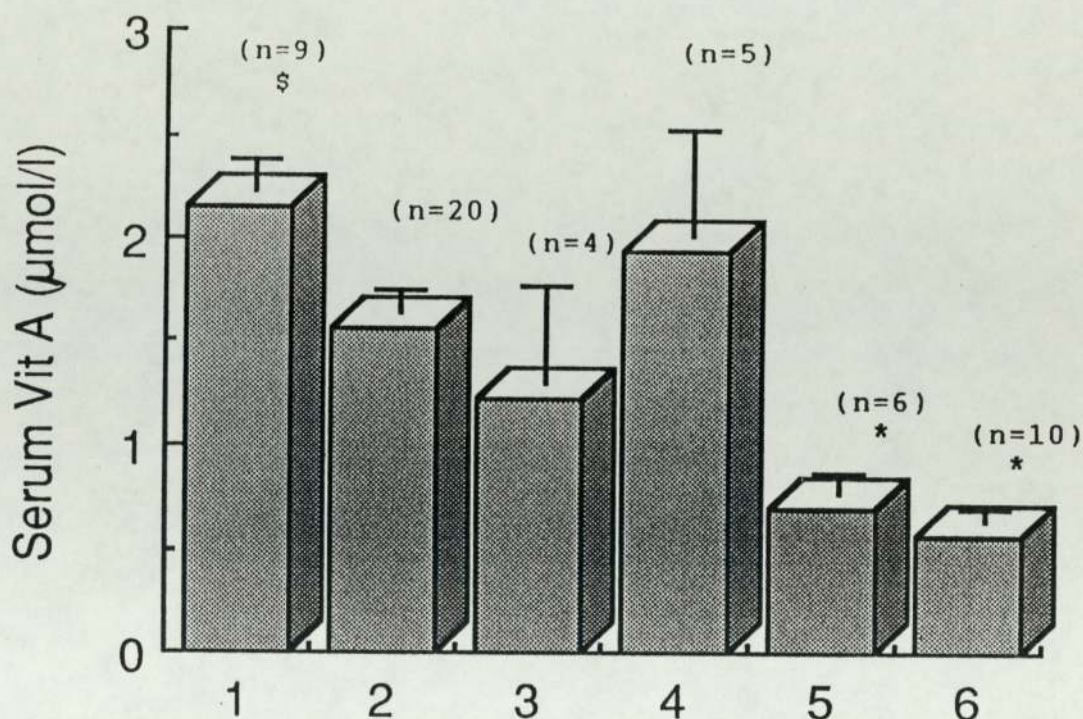
Where:

1	=	Adult volunteers
2	=	Liver transplant recipients
3	=	Patients with cholestasis associated with parenteral feeding
4	=	Patients with glycogen storage disease
5	=	Patients with neonatal hepatitis
6	=	Patients with EHBA

*	$p < 0.001$
$	$p < 0.05$

Figure 6.2. Histogram representing serum vitamin A levels in chronic liver disease, prior to vitamin A supplementation. The abscissa shows patients as described by diagnosis, whilst the ordinate shows serum vitamin A levels (μmol/l). Histobars represent mean serum vitamin A level, with the central vertical bar giving the SEM. The number of specimens has been given in parenthesis. Serum vitamin A levels were found to be significantly lower in patients with neonatal hepatitis and EHBA when compared with liver transplant recipients ($p < 0.001$).

-149-

For comparison, the data available for patients, prior to and following supplements, has been shown in Table 6.2.

Group	n	Unsupplemented patients Serum vit A Mean (SEM) (μmol/l)	n	Patients + 2,500iu Serum vit A Mean (SEM) (μmol/l)
Post Liver Transplant	4	1.56 (0.11)	4	1.34 (0.11)
EHBA	10	0.57 (0.06)	56	0.79 (0.07)[1]*
			22	1.13 (0.08)[2]
Neonatal Hepatitis	6	0.70 (0.10)	17	1.28 (0.13)$

*   p < 0.001
$ p < 0.05

[1] Patients in whom Kasai operation was unsuccessful.
[2] Patients in whom Kasai operation was successful.

Table 6.2. Serum vitamin A levels in unsupplemented patients and in patients receiving 2,500iu of vitamin A each day.

Supplementation with 2,500iu (418±66iu/kg) of vitamin A resulted in significantly higher serum vitamin A levels in patients with neonatal hepatitis (p<0.05), the serum vitamin A levels in the supplemented patients being equivalent to those found in the liver transplant recipients.

Conversely, vitamin A levels in supplemented patients with EHBA following an unsuccessful Kasai operation (482±49iu/kg) (p<0.001), cirrhosis (214±33iu/kg) (p<0.001) and alpha$_1$-antitrypsin deficiency (357±71iu/kg) (p<0.05)

Serum Vit A (umol/l)

(n=11)
(n=22)
(n=56) *
(n=15) *
(n=17)
(n=7) $

2

1

0

1   2   3   4   5   6
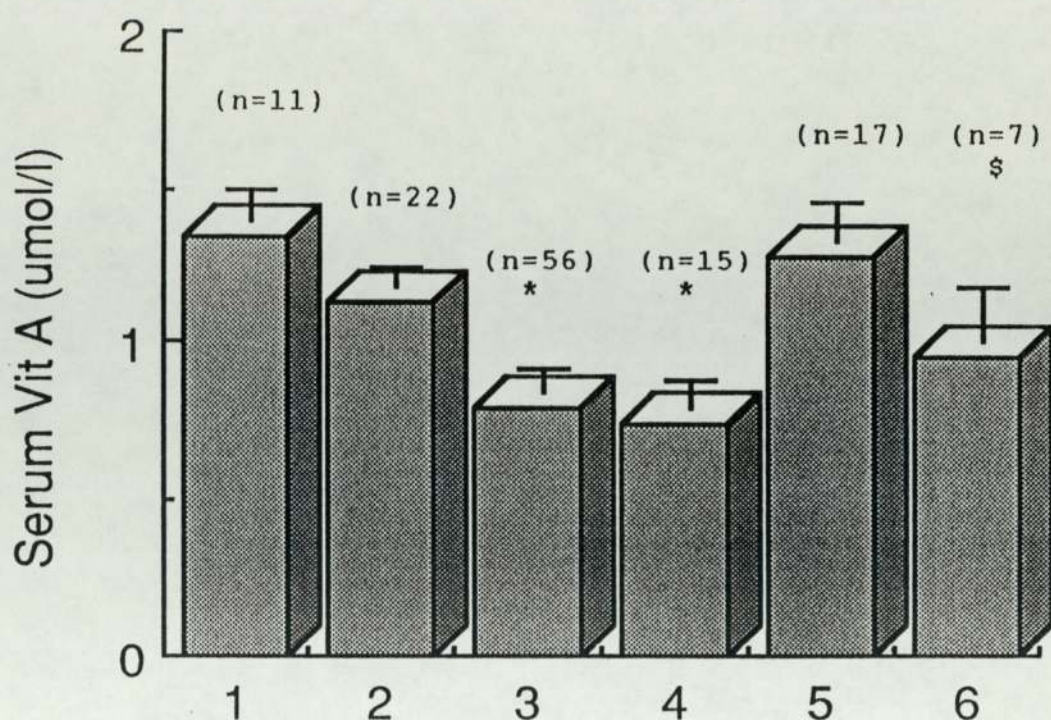
Where:

1	=	Liver transplant recipients
2	=	Patients with EHBA, following a successful Kasai operation
3	=	Patients with EHBA, following an unsuccessful Kasai operation
4	=	Patients with cirrhosis
5	=	Patients with neonatal hepatitis
6	=	Patients with alpha$_1$-antitrypsin deficiency

*   p < 0.001
$ p < 0.05

Figure 6.3. Histogram representing serum vitamin A levels in chronic liver disease, following oral supplementation with 2,500iu of vitamin A each day. The abscissa shows patients as described by diagnosis, whilst the ordinate shows serum vitamin A levels (μmol/l). Histobars represent mean serum vitamin A level, with the central vertical bars giving the SEM. The number of specimens has been given in parenthesis. Serum vitamin A levels were significantly lower in EHBA following an unsuccessful Kasai operation (p<0.001), cirrhosis (p<0.001) and α_1-antitrypisn deficiency (p<0.05) when compared with liver transplant recipients.

-151-

remained significantly lower than those obtained from liver transplant recipients. However, serum vitamin A levels in patients with EHBA (receiving 186 ± 13iu/kg of vitamin A/day) following a successful Kasai operation were not significantly different from those in the liver transplant recipients.

If the groups of patients receiving vitamin A supplements were compared with each other, 2 distinct groups were observed (Figure 6.4). Patients with EHBA following an unsuccessful Kasai operation or cirrhosis had similar low serum vitamin A levels, whilst patients with EHBA following a successful Kasai operation, or neonatal hepatitis had significantly higher serum vitamin A levels ($p<0.05$). The patients with $alpha_1$-antitrypsin deficiency had a mean serum vitamin A level which fell between the 2 groups.

3. The effect of increasing oral vitamin A dosage on serum vitamin A levels in EHBA.

The most severe biochemical vitamin A deficiency ($0.57\pm0.06\mu$mol/l, n=10) was observed in patients with EHBA where the Kasai operation had been unsuccessful. Oral supplementation with 2,500iu of vitamin A did not result in any significant elevation of serum vitamin A levels, therefore the daily dose of vitamin A was increased in 7 patients, in order to establish whether elevation of the serum levels was possible. Dosage increases were arbitrary,

-152-
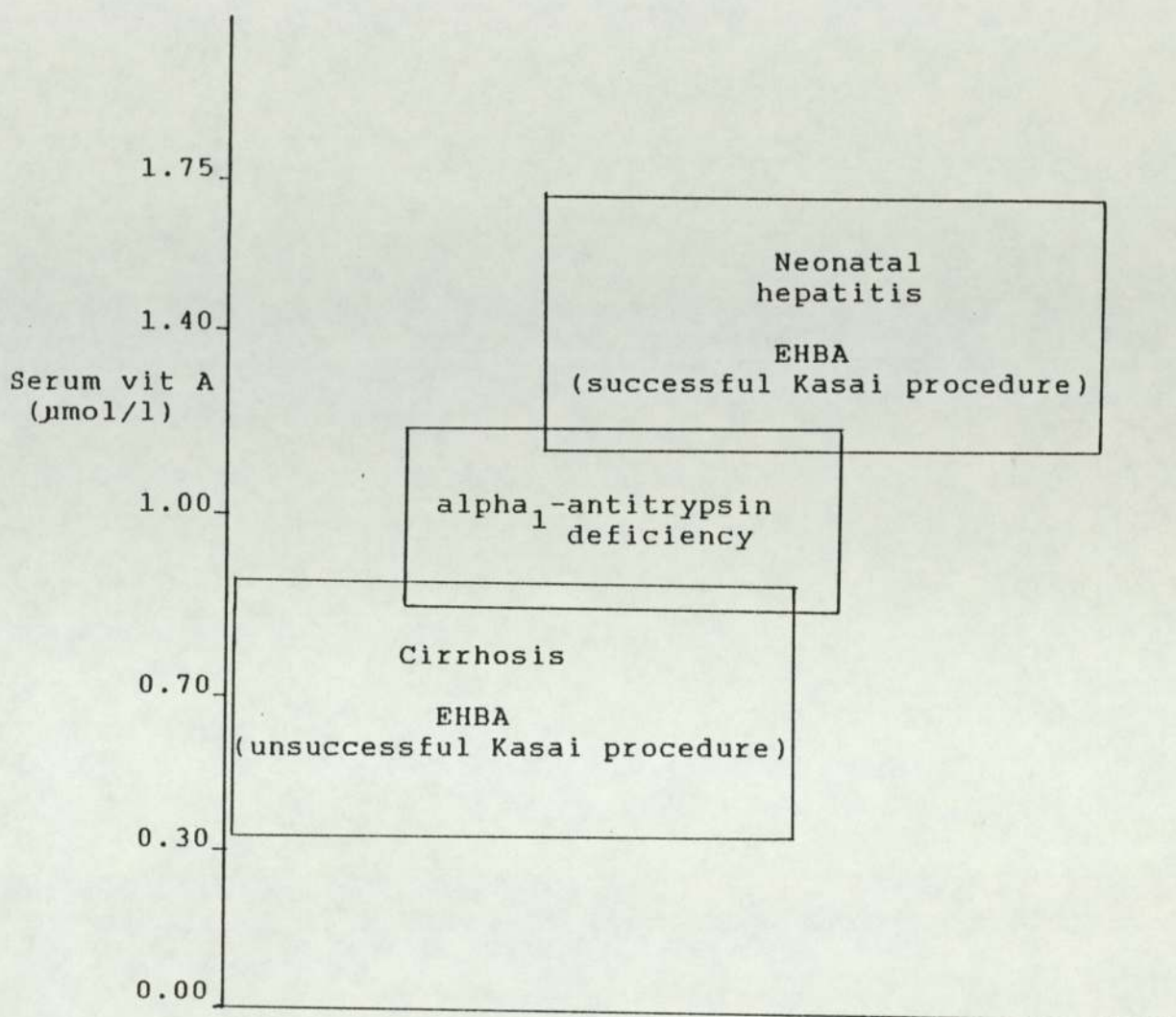
Figure 6.4. A graph showing 2 distinct groups of patients
with chronic liver disease classified according to serum
vitamin A levels. Serum vitamin A levels were significantly
higher in patients with neonatal hepatitis where jaundice
was resolving, and EHBA where Kasai operation had been
successful, when compared with patients in whom the Kasai
operation had been unsuccessful or who had cirrhosis. Serum
vitamin A levels in α_1-antitrypsin deficiency fell in
between the 2 groups.

based on the response of serum vitamin A levels to the previous dose of vitamin A.

The 7 patients, when supplemented with 2,500iu of vitamin A had a mean serum vitamin A level of $0.56\pm0.07\mu$mol/l (n=23), and therefore fell within the study population of patients presenting with an unsuccessful Kasai operation. Since different doses of vitamin A were administered to each patient, they have been considered separately.

An increase in the daily oral supplement to 15,000iu of vitamin A resulted in elevation of serum vitamin A levels ($1.53\pm0.18\mu$mol/l, n=5) in one of the patients. The increase in serum vitamin A level was associated with the development of bile drainage, and therefore clinical improvement. Despite doses of up to 27,500iu (2,200iu/kg) of vitamin A, the remaining 6 patients did not respond, however the clinical condition deteriorated in all of these patients.

iv) Serum vitamin A levels in cystic fibrosis.

There was a significant difference ($p<0.05$) in the mean serum vitamin A levels obtained from the 1-4 year old patients ($1.47\pm0.09\mu$mol/l, n=20) and the 8-18 year old patients ($1.21\pm0.05\mu$mol/l, n=58) with cystic fibrosis. Moreover, the serum vitamin A levels in both groups were significantly lower than those of the adult controls ($p<0.001$), but not the liver transplant recipients (Figure 6.5).

-154-

Serum Vit A (µmol/l)

3

2

1

0

(n=9)
$

(n=11)

(n=58)

(n=20)

1   2   3   4
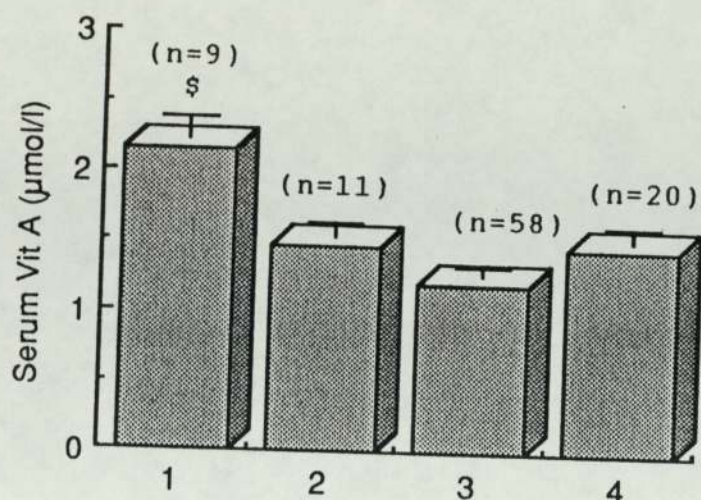
Where:

1   =   Adult controls
2   =   Liver transplant recipients
3   =   Patients with cystic fibrosis aged 8-18 years
4   =   Patients with cystic fibrosis aged 1-4 years

$ p< 0.05

Figure 6.5. Histogram representing serum vitamin A levels in cystic fibrosis. The abscissa shows patients with cystic fibrosis divided by age into 2 groups (8-18 years & 1-4 years) (3 & 4), compared with adult controls (1) and liver transplant recipients (2). The ordinate shows serum vitamin A levels (umol/l) and histobars represent mean serum vitamin A level, with central vertical bars giving the SEM. Serum vitamin A levels in cystic fibrosis were significantly lower than the adult controls (p<0.001) but not the liver transplant recipients. Furthermore as age increased in cystic fibrosis, so serum vitamin A levels fell (p<0.05).

v)   Serum vitamin A levels in gastrointestinal disease.

The   serum vitamin A levels obtained from each group of patients with gastrointestinal disease have been summarised in **Figure** 6.6.   Although   gastrointestinal   disease   was associated with a significantly lower mean serum vitamin A level (1.32±0.11μmol/l,   n=23)   than   the   adult   controls (p<0.001), there was no significant difference when compared with the liver transplant recipients.

This finding was duplicated in each group of patients, except those with coeliac disease where serum vitamin A levels were not significantly different from those found   in the adult controls.

vi)   Distribution of serum vitamin A levels.

Since transient falls in serum vitamin A levels were anticipated in a normal population, interpretation of the single measurements obtained from the patients with. cystic fibrosis and gastrointestinal disease in the present study was difficult. Each group of patients was therefore assessed to determine what proportion of each population had serum levels less than 1.0μmol/l whilst receiving standard vitamin A supplements (see chapter 3.i). Patients with chronic liver disease, for whom multiple determinations were available were only included if the mean serum vitamin A level was less than 1.0μmol/l. The distribution of serum vitamin A levels within each group of patients has been summarised in **Figure** 6.7.
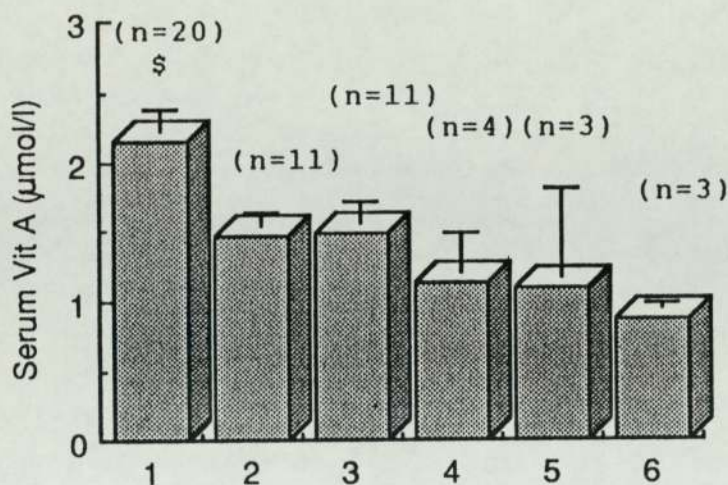
Where:

1 = Adult controls
2 = Liver transplant recipients
3 = Patients who were failing to thrive
4 = Patients with short bowel syndrome
5 = Patients with coeliac disease
6 = Patients with malabsorption

$ $p < 0.05$

.Figure 6.6. Histogram representing serum vitamin A levels in
gastrointestinal disease. The abscissa shows patients as
described by diagnosis, whilst the ordinate shows serum
vitamin A levels (µmol/l). Histobars represent mean serum
vitamin A levels with the central vertical bar giving the
SEM. The number of specimens has been given in parenthesis.
Patients with malabsorption were undergoing investigation
for anorexia and weight loss. Patients with gastrointestinal
disease except those with coeliac disease had serum vitamin
A levels significantly lower than the adult controls
(p<0.05) but not the liver transplant recipients.

1.   Chronic liver disease.

Persistently low serum vitamin A levels were found in
patients with chronic liver disease, despite supplementation
with 2,500iu of vitamin A. In EHBA, 80% of the patients had
low serum vitamin A levels ($0.44\pm0.05\mu$mol/l, n=12) following
an unsuccessful Kasai operation, whilst only 18% of those
with a successful Kasai operation had persistently low serum
vitamin A levels (0.75μmol/l, n=2). However, 36% of the
latter group did experience a transient drop in serum
vitamin A level.

Moreover, low serum vitamin A levels ($0.59\pm0.15\mu$mol/l
(n=6) were found in 4 out of 5 (80%) patients with neonatal
hepatitis who were not receiving vitamin A supplements.
However, only 1 of the 8 (12.5%) patients with neonatal
hepatitis who were receiving a daily oral supplement of
2,500iu of vitamin A had persistently low serum vitamin A
levels   ($0.59\pm0.08\mu$mol/l,   n=3),   indicating   that
supplementation was successful in these patients.

In alpha$_1$-antitrypsin deficiency and glycogen storage
disease only 1 of each of the 5 patients demonstrated low
serum vitamin A levels ($0.48\pm0.05\mu$mol/l, n=5 and 0.53μmol/l
respectively). Both patients with chronic active hepatitis
also had low serum vitamin A levels ($0.65\pm0.02\mu$mol/l, n=9).

-158-
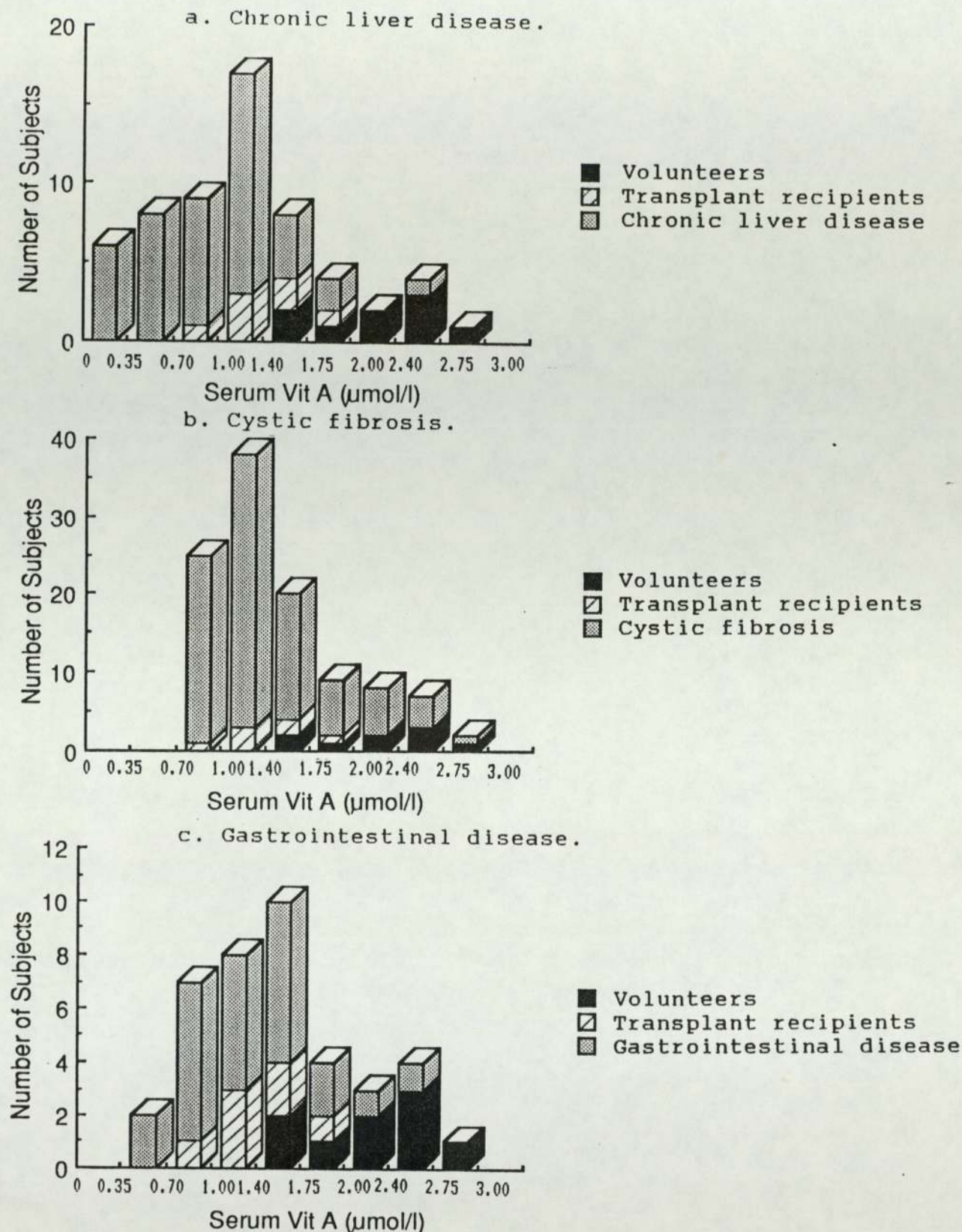
Figure 6.7. Histograms representing the distribution of
serum vitamin A levels in chronic liver disease (a.),
cystic fibrosis (b.) and gastrointestinal disease (c.),
compared with adult controls and liver transplant
recipients. The abscissa shows serum vitamin A levels
(umol/l), whilst the ordinate shows numbers of subjects.
Although overlap with adult controls and transplant
recipients was noted, serum vitamin A levels tended towards
the lower end of the scale in chronic liver disease and
gastrointestinal disease. In contrast, serum vitamin A
levels in cystic fibrosis showed a similar distribution to
those of the liver transplant recipients.

2.   Cystic fibrosis.

Of the patients with cystic fibrosis, 26% of the 1-4 year old patients and 25% of the 8-18 year old patients were found to have serum vitamin A levels less than 1.0μmol/l (0.78±0.05μmol/l, n=12 and 0.82±0.03μmol/l, n=12, respectively), despite supplementation. Measurement of serum vitamin A levels was repeated on 7 of the 8-18 year old patients a year after the first analysis, and serum levels remained low (0.76±0.05μmol/l) (n=7). These findings indicated that the serum vitamin A levels were likely to be relatively consistent.

3.   Gastrointestinal disease.

Serum vitamin A levels were found to be less than 1.0μmol/l in 8 patients (35%) with gastrointestinal disease (3 with malabsorption, 1 coeliac disease, 2 short bowel syndrome, 2 failure to thrive).

This approach was extended to consider those patients with serum vitamin A levels < 0.7μmol/l and < 0.35μmol/l, since these serum levels have been associated with subclinical and clinical vitamin A deficiency respectively (Amedee-Manesme et al., 1985, Underwood, 1978).

The results have been summarised in Table 6.3. No patients with cystic fibrosis had serum vitamin A levels < 0.35μmol/l, and only 6-9% demonstrated serum levels

< 0.7μmol/l. However, 30% of the patients with chronic liver disease had serum vitamin A levels < 0.7μmol/l, with 9% < 0.35μmol/l. When the patients with EHBA following an unsuccessful Kasai operation were considered separately, these figures increased to 73% < 0.7μmol/l and 27% < 0.35μmol/l.

Group	(n)	Serum Vit A <0.35μmol/l		Serum Vit A <0.70μmol/l	
		(n)	(%)	(n)	(%)
Cystic Fibrosis					
8-18 years	(48)	0	0	3	6
1-4 years	(46)	0	0	4	9
Chronic Liver Disease	(44)	4	9	13	30
EHBA (unsuccessful Kasai operation)	(15)	4	27	11	73
EHBA (successful Kasai operation	(11)	0	0	0	0

Table 6.3. Patients with serum vitamin A levels < 0.70μmol/l and < 0.35μmol/l.

-161-

vii)   The relationship between serum vitamin A levels and
        dietary intake of vitamin A.

1.   The effect of dietary intake of vitamin A on serum
        levels.

        A 7-day dietary assessment was completed by the
parents of 12 patients (8 male, 4 female) with chronic liver
disease (2.94±1.22 years, ranging from 0.25 to 14 years).
The data was analysed using the Microdiet computor program
(University of Salford) which calculated the daily intake of
vitamin A-active foods (carotenoids and vitamin A esters),
including any vitamin A supplements, and compared this with
the recommended daily intake (RDI) (WHO, 1967). The dietary
· intake of vitamin A-active foods was determined as a
percentage of the recommended daily vitamin A intake for
each patient, taking into account the age of the patient.
The results have been summarised in Table 6.4.

        Only 3 patients in the present study had a dietary
intake of vitamin A < 100% of the recommended daily intake.
Following vitamin A supplementation, the intake of vitamin
A active foods was < 100% of the recommended daily intake
in only 1 of the 12 patients. There was no correlation
between serum vitamin A level (0.90±0.14μmol/l, n=12) (range
0.22-1.08μmol/l) and intake of vitamin A active foods.

Subject	RDI (µg)$	Vit A intake (ug)	Vit A supplement* (µg)	Total intake (%RDI)	Serum Vit A (µmol/l)
1	450	750	3000	834	0.83
2	575	450	750	208	1.40
3	725	525	——	72	0.87
4	450	472	——	105	0.87
5	450	611	——	136	0.33
6	450	720	——	160	0.76
7	300	520	750	423	0.22
8	450	362	750	247	1.06
9	300	998	7,500	2833	0.95
10	450	1065	——	237	0.24
11	300	575	750	442	1.45
12	450	474	2,250	605	1.80

$ Calculated on intake per kg body weight
* µg = iu x 0.3

Table 6.4. Daily intake of vitamin A-active foods & supplements in chronic liver disease.

2.   The effect of intensive nasogastric feeding on serum
      vitamin A levels.

The nutritional status of 9 patients was improved with
intensive enteral feeding (Charlton et al., 1988). Despite
significant improvement in dietary intake and anthropometric
measurements (p<0.05), serum vitamin A levels did not
increase from $0.87\pm0.10\mu mol/l$ (n=9).

viii)   The relationship between serum vitamin A levels and
         liver function.

1.   Standard liver function tests.

Serum levels of vitamin A were compared with the
routine liver function tests determined at The Children's
Hospital, Birmingham (Appendix ii.), in order to establish
whether there was a correlation between serum vitamin A
levels and any of the routine liver function tests. In
particular, serum albumin levels were taken as indicative of
the ability of the liver to synthesise transport proteins,
including RBP. Serum bilirubin levels were taken as
indicative of the degree of cholestasis. The serum levels
were determined on 50 specimens taken from 23 patients with
chronic liver disease. No significant correlation between
serum vitamin A levels and any of the liver function tests
was found.

2.   Caffeine clearance

Serum vitamin A levels were also compared with the elimination half-life for caffeine in 55 patients with chronic liver disease, who had been given 3mg/kg of caffeine (as caffeine citrate) orally (Baker et al., 1988). The elimination half-life of caffeine ($t_{1/2}$) and serum vitamin A levels obtained from each group of patients have been summarised in Figure 6.8. Since there was no significant difference in the caffeine half-life found in adult controls and liver transplant recipients, the two groups were combined and used as controls.

In chronic liver disease, the caffeine half-life was found to correlate with the severity of the disease state ranging form 1.6 to 3.5 times greater than the controls ($p<0.05$). There was no correlation between individual serum vitamin A or carotenoid levels and caffeine half-life, however mild or moderate-severe liver disease resulted in significantly lower serum vitamin A levels ($p<0.05$). No distinction could be made between those with moderate or severe disease.

c.   Severity of liver disease.

A further 23 patients (137 specimens) were classified as having mild, moderate, or severe liver disease (See section xi.7.) (Baker et al., 1988). This classification system utilised four paramaters of liver function, rather

-165-
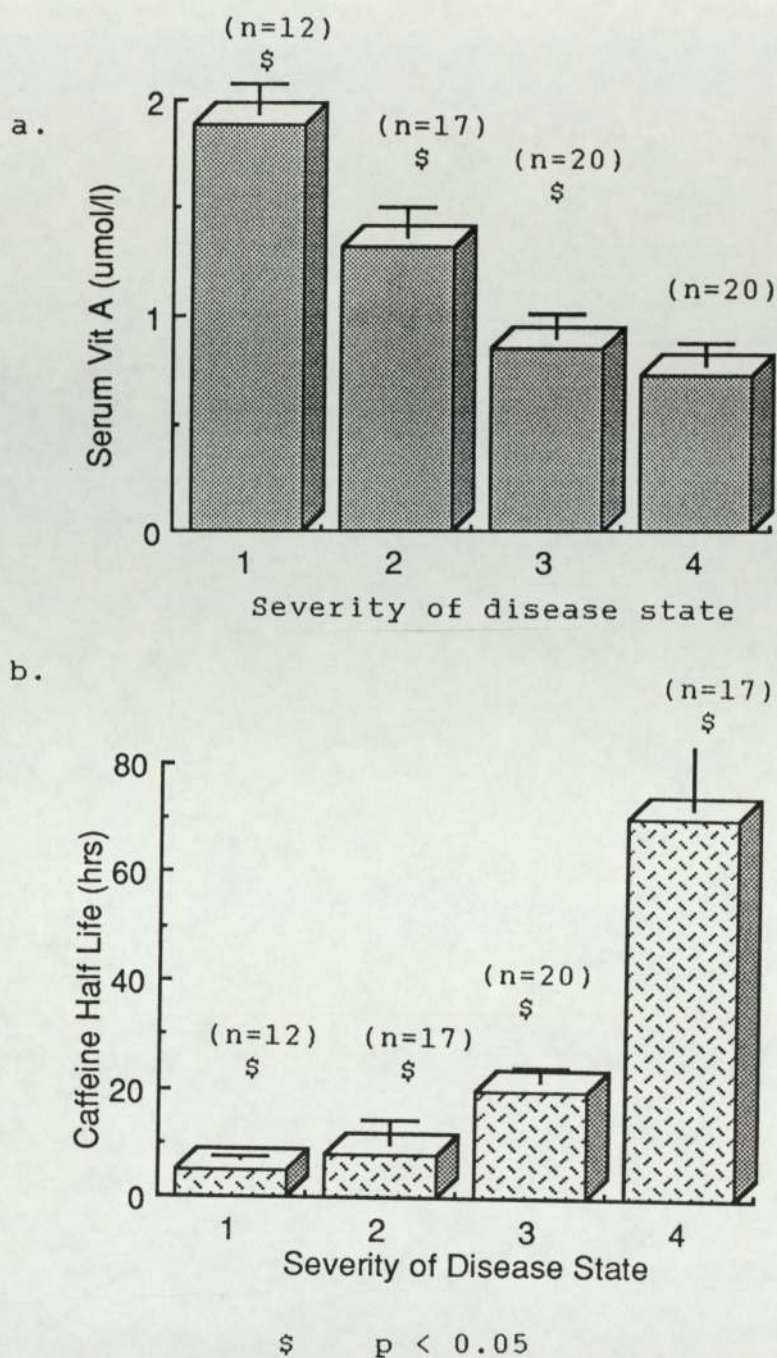
Figure 6.8. Histograms representing corresponding serum
vitamin A levels and caffeine elimination half-life in
chronic liver disease. The abscissa shows the severity of
disease, ranging from mild (2) to severe (4), compared with
controls (1). The ordinate shows serum vitamin A level (a.)
or caffeine half-life following ingestion of 3mg/kg of
caffeine (as caffeine citrate) (b.). Histobars represent
mean value with the central vertical bar giving the SEM.
Mild and moderate liver disease were associated with
significantly lower serum vitamin A levels (p<0.05), however
severe liver disease did not cause further fall in serum
levels. Caffeine half-life did correlate with the severity
of liver disease (p<0.05).

than single liver function tests to assess the disease severity. Mild and moderate-severe liver disease were found to result in significantly lower serum vitamin A levels than the controls ($p < 0.05$), although no distinction could be made between serum vitamin A levels in moderate and severe liver disease, confirming the findings described above.

ix) The relationship between vitamin A and vitamin E.

1. In chronic liver disease.

Serum levels of vitamin A and Vitamin E were determined simultaneously on 14 specimens, taken from 12 patients (8 male, 4 female) with chronic liver disease, in order to establish whether there was any correlation between the serum levels of the two vitamins. Of the 12 patients, 5 had EHBA (only one had a successful Kasai operation), whilst 2 had idiopathic cirrhosis. A further 3 patients suffered from neonatal hepatitis, and the remaining 2 patients had either glycogen storage disease or $alpha_1$-antitrypsin deficiency.

There was no correlation between serum levels of vitamin A ($0.95 \pm 0.14 \mu mol/l$, n=14) and vitamin E ($16.63 \pm 4.44 \mu mol/l$, n=14). Although all patients received 15mg of vitamin E each day (as Ketovite tablets) the daily dose of vitamin A ranged from 2,500iu to 25,000iu, therefore the lack of correlation could result from the use of different doses of vitamin A supplements. However, there was

-167-

no correlation between the oral dose of vitamin A and the serum levels.

Despite the lack of correlation between serum levels of the 2 vitamins, serum vitamin E levels were found to be significantly lower in EHBA (7.30 ± 2.49µmol/l, n=7) compared with the other causes of liver disease (25.83 ± 12.05µmol/l, n=7) (p<0.05), indicating that patients with severe liver disease were at greater risk of developing both vitamin A and vitamin E deficiency. However, all patients except 3 with EHBA were found to have serum vitamin E levels just within the lower end of the normal range (6-30µmol/l).

2.   In cystic fibrosis.

Data was also available from 28 patients with cystic fibrosis (aged 12-18 years). These patients maintained adequate serum vitamin A levels (1.31 ± 0.09µmol/l, n=28), however the serum vitamin E levels (6.35 ± 1.08µmol/l, n=28) were low, with only 7 of the patients demonstrating serum vitamin E levels within the normal range.

x)   The relationship between vitamin A and zinc.

Since zinc is required for retinol-binding protein (Smith et al., 1973b) and pre-albumin synthesis (Bates & McClain, 1981), Smith and co-workers (1976) suggested that there was a correlation between serum vitamin A and zinc levels.

Furthermore, zinc may be involved in the enzymatic conversion of vitamin A alcohol to the aldehyde in the retinal rods (Russell et al., 1980). Low serum levels of zinc have been reported in cystic fibrosis, liver disease (Smith et al., 1973, Brissot et al., 1978) and gastrointestinal disease (Main et al., 1983).

The serum zinc levels obtained in the present study, from patients with chronic liver disease ($9\pm1\mu$mol/l, n=15) and cystic fibrosis ($8\pm2\mu$mol/l, n=26) fell just below the lower limits of the normal range (10-20μmol/l). There was no correlation between serum vitamin A ($0.89\pm0.11\mu$mol/l, n=15 & $1.31\pm0.0.09\mu$mol/l, n=26, respectively) and zinc levels in each group. Unfortunately serum retinol-binding protein levels were not available.

xi)   Discussion

In the present study, serum vitamin A levels were found to be within the normal range in adult controls and liver transplant recipients (Eastham, 1985, Letner, 1984). Moreover, patients with gastrointestinal disease and cystic fibrosis, who were receiving vitamin A supplements also maintained adequate serum vitamin A levels. However, serum vitamin A levels fell when the dose and preparation of vitamin A was changed from a water-miscible liquid to a capsule in the older patients with cystic fibrosis. Conversely, supplementation with a daily dose of 2,500iu of

vitamin A did not maintain serum vitamin A levels in a number of patients with chronic liver disease. Unfortunately increased dosage did not result in elevation of serum vitamin A levels in patients with EHBA following an unsuccessful Kasai operation, in whom biochemical vitamin A deficiency was found to be most severe.

1.   Serum vitamin A levels in healthy individuals.

The serum vitamin A levels obtained from the adult controls were found to be within the published normal ranges (Eastham, 1985, Lentner, 1984). However lower serum vitamin A levels were expected in children under the age of 10 years (Lentner, 1984, O'Neil et al., 1970, High, 1969). A newborn full term infant for example, might have a mean serum vitamin A level as low as $0.77\mu mol/l$ (Lentner, 1984), increasing rapidly to $1.0\mu mol/l$ (O'Neil et al 1970, High 1969). Adult levels have been reached by the age of 10-13 years (Lentner, 1984).

Since age-matched normal controls could not be obtained for the present study, the liver transplant recipients were used as controls (see below). There was no evidence to suggest that these patients should have abnormal vitamin A status, except during episodes of rejection of the donor liver or prolonged/recurrent infection (Sivakumar & Reddy, 1972); in these circumstances specimens were excluded from the present study. Furthermore, the liver transplant

recipients were only included as controls in the present study at least 28 days following the liver transplant, when serum bilirubin ($14\pm2\mu$mol/l, n=20) and albumin levels (32 ± 3g/l, n=20) were stable within the normal range for age (see appendix ii). The serum vitamin A levels obtained from the liver transplant recipients were found to be within the expected normal range for age (Lentner, 1984, O'Neil et al., 1970).

2.   Serum vitamin A levels in liver transplant recipients.

Biochemical vitamin A deficiency was not anticipated or observed in liver transplant recipients, since vitamin A in the donor liver could be utilised to maintain serum vitamin A levels. Furthermore, any defect in retinol-binding protein synthesis resulting from the presenting disease state would presumably be corrected following the liver transplant. Moreover, vitamin A intake should increase since patients would no longer malabsorb vitamin A.

Serum vitamin A levels were not determined for at least 28 days following liver transplantation owing to clinical priorities, therefore it was impossible to establish how rapidly serum vitamin A levels were restored following a successful liver transplant. Serum vitamin A levels fell in only one patient following the liver transplant. Serum specimens from this patient were noted to be very lipidaemic at the time of analysis prior to liver transplantation, and

previous studies indicated that interference might have occurred during analysis (Underwood, 1984).

Supplementation with 2,500iu (334±102iu/kg) of vitamin A did not appear to be necessary to maintain adequate serum vitamin A levels in the liver transplant recipients. Presumably, any supplemented vitamin A would be transported to the liver for storage, rather than being immediately mobilised since serum vitamin A levels had been maintained by vitamin A stored in the donor liver (Peterson et al., 1973). These findings could be substantiated by withdrawal of supplements in the four patients receiving them, with further monitoring on serum vitamin A levels.

3.   Serum vitamin A levels in chronic liver disease, prior to supplementation.

Many of the patients included in the present study were referred from other hospitals, therefore data on the serum vitamin A levels prior to supplementation was only available for a limited number of patients with chronic liver disease. Patients with glycogen storage disease, cholestasis associated with parenteral feeding, Wilson's Disease and hyperlipidaemia did not require vitamin A supplementation to maintain adequate serum vitamin A levels. There was no evidence of chronic liver disease in the patients with cholestasis or hyperlipidaemia, whilst the patient with Wilson's Disease was well controlled, therefore normal

-172-

vitamin A status would be expected. Although two of the patients with glycogen storage disease were poorly controlled, and one had a grossly enlarged liver resulting from deposition of glycogen, serum vitamin A levels were not reduced except in a third patient (0.53μmol/l). Conversely, much lower serum vitamin A levels were found in the patients with a more severe form of chronic liver disease such as cirrhosis or EHBA. These results indicated that a relationship between serum vitamin A levels and the severity of the liver disease might occur.

4.   Serum vitamin A levels in patients with chronic liver disease who were receiving vitamin A supplements.

In retrospect, a daily oral vitamin A supplement of 2,500iu (347 ± 29iu/kg) appeared to be adequate in patients with neonatal hepatitis in whom cholestasis was resolving, and patients with EHBA following a successful Kasai operation. However, at the time of initial cholestasis and therefore introduction of vitamin A supplements, the clinical outcome of the condition was impossible to predict. Supplementation with 2,500iu of vitamin A was not sufficient to maintain adequate serum vitamin A levels in patients with EHBA where the Kasai operation had been unsuccessful (482 ± 49iu/kg), in cirrhosis (214 ± 33iu/kg) or in $alpha_1$-antitrypsin deficiency (357 ± 71iu/kg).

Dosage requirements for vitamin A in EHBA probably vary considerably, but supplementation with an oral daily dose of 2,500iu of vitamin A resulted in the elevation of serum vitamin A levels only in patients who had a successful Kasai operation. These patients had achieved bile drainage, and whilst there might have been some evidence of cirrhosis this did not appear to cause clinical problems at the time of analysis.

In patients with EHBA following an unsuccessful Kasai operation, a suitable dose of vitamin A was difficult to establish other than by trial and error, since there was large variation in the clinical condition of individuals, and the disease state was often rapidly progressive. Initial doses of vitamin A should probably be far in excess of 2,500iu (482$\pm$49iu/kg) since doses of up to 27,500iu vitamin A failed to elevate serum vitamin A levels, except in one patient where there was clear evidence of clinical improvement.

Measurement of hepatic vitamin A concentration in patients with chronic liver disease would allow determination of any hepatic accumulation of vitamin A, resulting from absorption of high doses vitamin A followed by defective transport, caused by defective RBP synthesis and/or release (see chapter 11.viii.) (Vahlquist et al., 1978). In these circumstances increasing the dose of vitamin A would be of little benefit since the vitamin could not

-174-

reach the sites of action. Furthermore, the serum
concentration of vitamin A esters is thought to increase in
the absence of retinol-binding protein, and these esters are
thought to cause toxicity as transport in serum is
unregulated. Several investigators have postulated that the
vitamin A esters cannot interact with the cell surface
receptors responsible for mediating the actions of vitamin
A, which are relatively specific for holo-RBP (vitamin A
complexed to retinol-binding protein) (Smith & Goodman,
1976, Vahlquist et al., 1982) (see chapter 2.vi).

5.   Serum vitamin A levels in cystic fibrosis.

As there have been reports of clinical vitamin A
deficiency in cystic fibrosis, patients should receive
vitamin A supplements (Raynor et al., 1988, Fulton et al.,
1982). The dosage regimen employed at The Children's
Hospital, Birmingham appeared to be adequate in the patients
with cystic fibrosis who were included in the present study,
however the significant fall in serum level with alteration
in the dose and preparation of vitamin A requires further
investigation. Absorption of vitamin A from a water-miscible
preparation such as Abidec drops or Ketovite liquid might be
more efficient than from a capsule preparation (Kalz &
Schafer, 1958), and continued administration of higher doses
of a water-miscible preparations of vitamin A might be
considered.

The drop in serum vitamin A levels, which occurred as age increased in cystic fibrosis might have been influenced by a number of factors. Firstly, the dose of vitamin A had been reduced and the pharmaceutical preparation changed from a water-miscible liquid to a capsule which is less readily absorbed (Kalz & Schafer, 1958). Secondly, the older patients with cystic fibrosis might suffer from more frequent infective exacerbations than the younger children, therefore serum vitamin A levels might be expected to be reduced (Littlewood, 1986, Sivakumar & Reddy, 1972). Although the drop in serum vitamin A levels was not great enough to indicate a high risk of subclinical vitamin A deficiency, adolescents with cystic fibrosis might undergo dark adaptation studies to establish the prevalence of clinical vitamin A deficiency, and the relationship with serum vitamin A levels in these patients.

6.   Serum vitamin A levels in gastrointestinal disease.

Serum vitamin A levels were significantly lower in patients with gastrointestinal disease (3.03 ± 0.89 years, n=23) when compared with the adult controls. However, this was probably related to age since there was no significant difference in serum vitamin A levels when compared with the age matched liver transplant recipients.

Supplementation with 2,500iu of vitamin A therefore appeared to be adequate to maintain serum vitamin A levels

in patients with gastrointestinal disease, unless malabsorption was known to be particularly severe. Although the 3 patients with malabsorption were found to have serum vitamin A levels less than 1.0µmol/l, these might have resulted from a transient fall in serum levels associated with an infection, since they were single measurements. Alternatively, these were older patients (9.89±2.16 years, n=3), who may have been suffering from malabsorption for a longer period of time. The serum vitamin A levels did however remain higher than those serum levels obtained from patients with chronic liver disease. There have been previous reports of subclinical vitamin A deficiency in gastrointestinal disease (Howard et al., 1982, Main et al., 1983), although much of the information appeared to be contradictory (Vahlquist et al., 1978, Imes et al., 1987).

7. The relationship between serum vitamin A levels and liver function in chronic liver disease.

In the present study, a vitamin A supplementation regimen based on the severity of liver disease was considered in patients with chronic liver disease, since the distribution of serum vitamin A levels indicated that serum vitamin A levels fell as the severity of liver disease increased (see iii above). No consistent relationship between serum vitamin A levels and the conventional liver function tests, particularly serum bilirubin and albumin levels was found in the present study.

-177-

The appearance of such a relationship might be hindered by the lack of sensitivity and specificity, not only in the measurement of serum vitamin A levels but also of the liver function tests themselves. Furthermore, consideration of individual liver function tests does not allow consideration of the time of onset, or the duration of the liver disease, or the influence of other factors such as recurrent infection (Sivakumar & Reddy, 1972).

Baker and co-workers (1988) devised a classification of liver disease, based on consideration of 4 major indicators of liver function. Thus patients could be classified as having mild, moderate, or severe liver disease (Table 6.5); fulfillment of one criterion in the moderate or severe classification resulted in inclusion in that group. Although such a classification was considered controversial, it did allow partial separation of patients with liver disease into reasonably well defined groups.

	Serum Albumin (g/l)	Serum Bilirubin (µmol/l)	Prolongation of PTT (seconds)	Ascites[*]
Mild	>35	<25	+ <12	−
Moderate	21-35	25-350	+ 12-20	−
Severe	<20	>350	+ >20	+

[*] Ascites was defined by clinical criteria.

Table 6.5. shows Baker and co-workers (1988) classification of liver disease, based on consideration of serum bilirubin & albumin levels, the prolongation of partial thromboplastin time (PTT) and presence of ascites (as defined by clinical criteria).

-178-

In the present study, consideration of more than one parameter of liver function was found to be necessary to investigate the role of chronic liver disease in the development of subclinical vitamin A deficiency, since no single parameter correlated with serum vitamin A levels. Therefore, the classification of Baker and co-workers (1988) was adopted. The relationship between this classification of liver disease and age might not be consistent, however the system was designed for, and only applied to children in the present study.

Although serum vitamin A levels did not appear to correlate with caffeine half-life, mild and moderate-severe liver disease, as described above did result in a significant fall in serum vitamin A levels. This confirmed the view that vitamin A requirements were related to severity of the disease state. Supplementation should therefore be tailored to the degree of severity of liver disease.

Whereas the findings in the present study established the need for flexible vitamin A supplementation, clarification is required before definite dosage recommendations could be made. The inability to distinguish between serum vitamin A levels from patients with moderate and severe liver disease might have resulted from the effect of duration, and time of onset of disease on vitamin A status. Hepatic stores of vitamin A may be sufficient to

-179-

maintain serum vitamin A levels for several months where onset of disease was relatively late (Robrigues & Irwin, 1972). Conversely, since hepatic vitamin A stores tend to be low at birth, early onset of liver disease might be associated with a more rapid decline in serum vitamin A levels. Moreover, similar hepatic stores might be found in a patient who had suffered from prolonged, moderate liver disease and a patient with an acute, severe episode of liver disease. Therefore time of onset and the duration of the disease state have to be considered along with the severity of liver disease in relation to vitamin A status.

The patients who underwent caffeine clearance tests were either receiving an oral vitamin A supplement of 2,500iu/day or no supplement. Since these patients were not receiving the same daily dose of vitamin A, any correlation between serum vitamin A level and caffeine half-life might be disguised by the varying vitamin A intake, however serum levels did not correlate with the daily vitamin A supplements.

However, the lack of correlation between individual determinations of caffeine half-life and serum vitamin A level, was more likely to result from the considerable variation in both parameters which was known to occur. In addition, although the classification of liver disease allowed consideration of 4 indices of liver function, other factors might be related to vitamin A status in these

-180-

patients, such as duration of the disease state (see above).

8.   The relationship between serum vitamin A and vitamin E
      levels.

No correlation was found between serum vitamin A and E levels in chronic liver disease. Despite the lack of correlation between the individual serum levels of vitamins A and E, serum levels of both vitamins were significantly lower in EHBA compared with other causes of liver disease. These findings indicated that severe liver disease was associated with a reduction in the serum levels of both vitamins, but that the magnitude of the fall in the serum levels was not consistent.

The low serum vitamin E levels found in both chronic liver disease and cystic fibrosis warrant further investigation. Although 11 patients with chronic liver disease were found to have serum vitamin E levels just within the lower limit of the normal range, many patients with EHBA might have low serum vitamin E levels and require vitamin E supplementation. Furthermore, only 7 of the patients with cystic fibrosis demonstrated a serum vitamin E level within the normal range, clearly indicating the need for vitamin E supplementation.

9.   The relationship between serum vitamin A and zinc
      levels.

Zinc is involved in several stages of vitamin A metabolism, retinol-binding protein and pre-albumin synthesis (Smith et al., 1973b, Bates & McClain, 1981). Furthermore, zinc may be involved in the storage and utilisation of vitamin A in the retinal rods (Russell, 1980). However the major effect of zinc on vitamin A metabolism appeared to result from a primary effect on appetite and food intake (Underwood, 1984).

Low serum zinc levels have been demonstrated in patients with cystic fibrosis, liver disease (Smith et al., 1973b, Brissot et al., 1978) and gastrointestinal disease (Main et al., 1983), however the reports appeared to be contradictory (Palin et al., 1979, Kaufman et al., 1987). Several investigators suggested that any zinc deficiency required correction if vitamin A supplementation was to be successful (Kaufman et al., 1987, Russell et al., 1973). Serum zinc levels in patients with chronic liver disease or cystic fibrosis in the present study fell just below the lower limit of the normal range. Further work is required to define the role of zinc in vitamin A metabolism, in order to appreciate the implications of these low serum zinc levels. A poor response to oral vitamin A supplements might be improved by concurrent administration of zinc supplements.

Although a correlation between serum zinc and vitamin A levels had previously been reported (Smith et al., 1976, Russell, 1980) no correlation between serum vitamin A and zinc levels was found in patients with chronic liver disease or cystic fibrosis in the present study.

vii) Summary.

The most severe biochemical vitamin A deficiency was associated with EHBA or cirrhosis, where the clinical prognosis was particularly poor. However, vitamin A status cannot be estimated on the basis of severity of disease alone, but may also be influenced by the time of onset and duration of the disease state.

Supplementation with the present regimens of a fixed dose of vitamin A appeared to be adequate in cystic fibrosis and gastrointestinal disease, unless malabsorption was particularly severe. However, continued supplementation with higher doses of a water-miscible vitamin A in cystic fibrosis requires investigation as the implications of the fall in serum vitamin A levels remained unclear. Furthermore, supplementation with a fixed daily dose of 2,500iu of vitamin A was also adequate in less severe forms of liver disease, such as neonatal hepatitis which resolved without evidence of cirrhosis. However the outcome of the disease could not be predicted at the time of diagnosis, therefore higher doses, or doses based on body weight might

be considered in chronic liver disease. Once the prognosis became clear or the clinical condition improved the dose would have to be adjusted accordingly to ensure that toxicity did not occur.

In the more severe forms of chronic liver disease, vitamin A requirements appeared to be much higher, therefore doses of vitamin A should be based on the severity of the disease, and calculated according to body weight. However, further investigation of the possible toxicity associated with high doses of vitamin A in the presence of defective retinol-binding protein synthesis or release requires investigation, before such an approach could be widely implemented.

CHAPTER 7: SERUM CAROTENOID LEVELS IN MALABSORPTIVE STATES.

i)   Serum carotenoid levels in adult controls.

The mean serum carotenoid level found in the adult controls in the present study, 1.74 ± 0.13mg/l (n=15), was just above the upper limits of the normal range (0.70-1.70mg/l) (High, 1969, O'Neil et al., 1970).

ii) Serum carotenoid levels in liver transplant recipients.

Lower serum carotenoid concentrations were found in the liver transplant recipients, when compared with the adult controls (Figure 7.1) ($p < 0.05$). Furthermore, liver transplant recipients receiving vitamin A supplements had a mean serum carotenoid level (0.56 ± 0.05mg/l, n=11) significantly lower than the unsupplemented liver transplant recipients (1.08 ± 0.16mg/l, n=20) ($p < 0.05$). Since the serum carotenoid levels in those liver transplant recipients who were receiving vitamin A supplements fell below the lower limits of the normal range (High, 1969), all subsequent groups of patients were only compared with the adult controls and those liver transplant recipients who were not receiving vitamin A supplements.

The serum carotenoid levels obtained from patients prior to, and following liver transplantation have been summarised in Table 7.1. There was no significant change in serum carotenoid level following liver transplantation or during episodes of rejection, except a fall in serum carotenoid level in one patient (patient 5, Table 7.1) following the liver transplant.

(n=15)

(n=20)
$

(n=11)
$

2

Serum Carotenoid (mg/l)

1

0

1   2   3
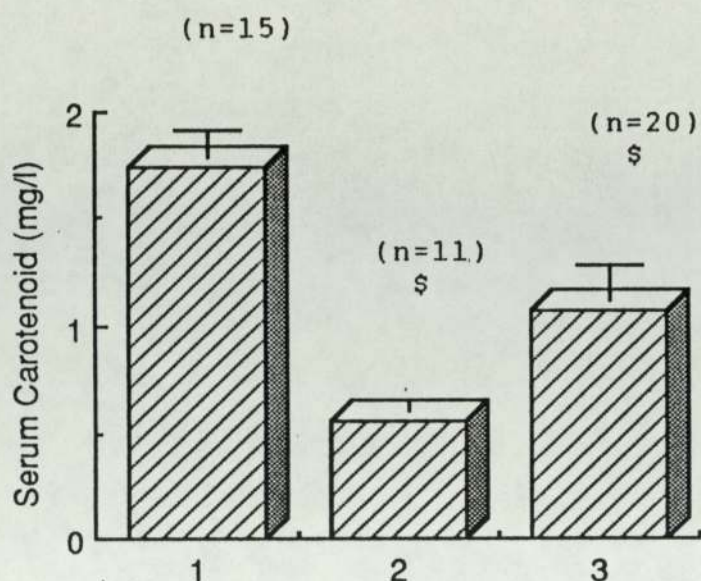
Where:

1   =   Adult controls
2   =   Liver transplant recipients who were not receiving
          oral vitamin A supplements
3   =   Liver transplant recipients receiving 2,500iu of
          vitamin A each day

$ p < 0.05

Figure 7.1. Histogram representing serum carotenoid levels
in liver transplant recipients. The abscissa distinguishes
between liver transplant recipients who were unsupplemented
(3) and those receiving 2,500iu of vitamin A each day (2),
compared with adult controls (1). The ordinate shows serum
carotenoid levels (mg/l). Histobars represent mean serum
carotenoid level, with central vertical bar giving the SEM.
The number of specimens have been given in parenthesis.
Serum carotenoid levels in liver transplant recipients were
significantly lower than in the adult controls (p<0.05).
Furthermore liver transplant recipients appeared to have
lower serum carotenoid levels whilst receiving vitamin A
supplements (p<0.05).

Patients	Pre Transplant				Post Transplant		
	(n)	Serum Carotenoid (mg/l)			(n)	Serum Carotenoid (mg/l)	
		Mean	(SEM)			Mean	(SEM)
1	(2)	0.38			(4)	1.42	(0.36)
2	(2)	1.72			(1)	0.72	
3	(2)	0.12			(5)	0.88	(0.16)
4	(3)	0.51	(0.23)		(4)	0.65	(0.24)
5	(7)	1.38	(0.24)		(5)	0.50	(0.13)

Table 7.1. Serum carotenoid levels pre- and post liver transplantation.

iii) Serum carotenoid levels in chronic liver disease.

The serum carotenoid levels obtained from each group of patients with chronic liver disease have been summarised in Figures 7.2 & 7.3. Although serum carotenoid levels were within the normal range, all groups except those with $alpha_1$-antitrypsin deficiency and glycogen storage disease were found to have had serum levels significantly lower than the adult controls ($p < 0.001$, $p < 0.005$ or $p < 0.05$). However, only those patients with glycogen storage disease had significantly different serum carotenoid levels from the unsupplemented liver transplant recipients ($p < 0.05$) (see section vi.3.). No correlation between serum vitamin A and carotenoid levels was observed in the present study.
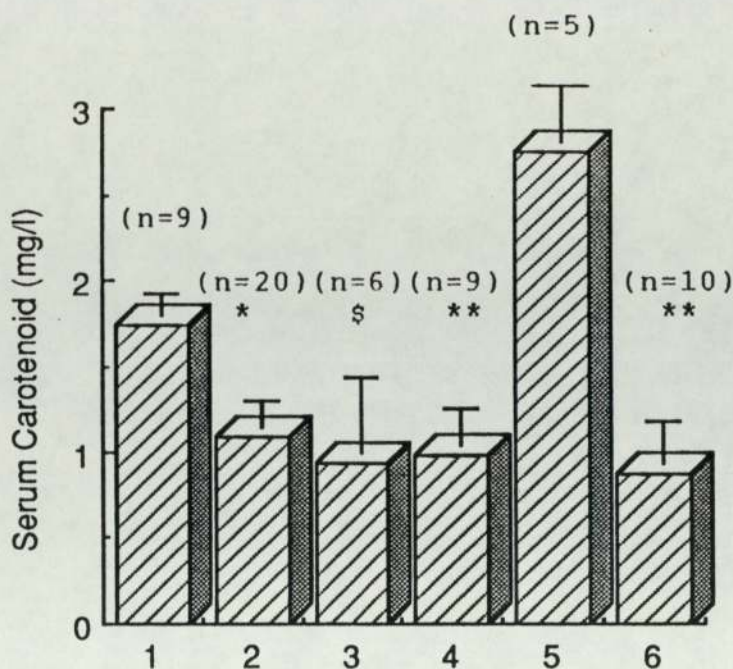
Where:

```
1    =    Adult controls
2    =    Liver transplant recipients
3    =    Patients with neonatal hepatitis
4    =    Patients with chronic active hepatitis
5    =    Patients with glycogen storage disease
6    =    Patients with EHBA
```

$$
\begin{array}{ll}
* & p < 0.001 \\
** & p < 0.005 \\
\$ & p < 0.05
\end{array}
$$

Figure 7.2. Histogram representing serum carotenoid levels in chronic liver disease, prior to vitamin A supplementation. The abscissa shows patients as described by diagnosis, whilst the ordinate shows serum carotenoid level (mg/l). Histobars represent mean serum level, with central vertical bar giving the SEM. The number of specimens have been given in parenthesis. All patients with chronic liver disease except those with glycogen storage disease were found to have serum carotenoid levels significantly lower than the adult controls (p<0.001, p<0.005, or p<0.05), however serum levels remained within the normal range for age.
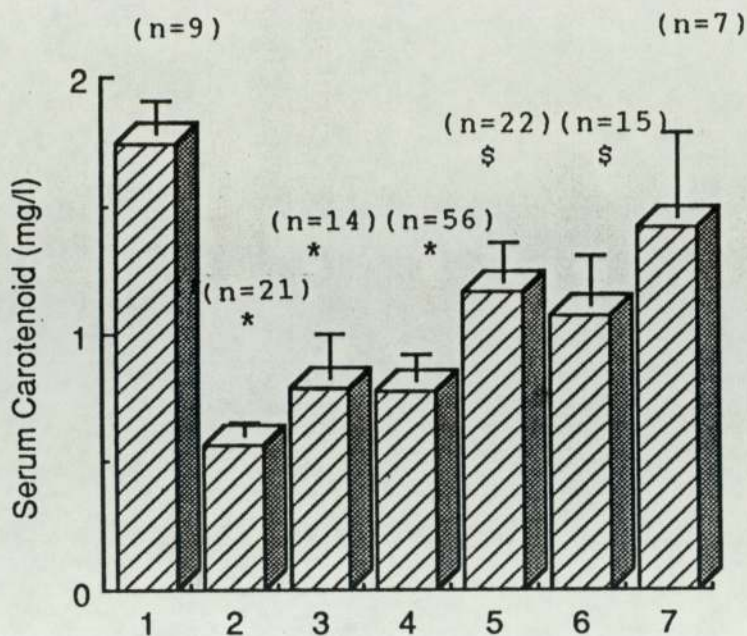
Where:

1	=	Adult controls
2	=	Liver transplant recipients
3	=	Patients with neonatal hepatitis
4	=	Patients with EHBA following an unsuccessful Kasai operation
5	=	Patients with EHBA following a successful Kasai operation
6	=	Patients with cirrhosis
7	=	Patients with alpha$_1$-antitrypsin deficiency

$$
\begin{array}{ll}
* & p < 0.001 \\
\$ & p < 0.05
\end{array}
$$

Figure 7.3. Histogram representing serum carotenoid levels in chronic liver disease, following supplementation with 2,500iu of vitamin A each day. The abscissa shows patients as described by diagnosis, whilst the ordinate shows serum carotenoid level (mg/l). Histobars represent mean serum carotenoid level, with the central vertical bar giving the SEM. The number of specimens have been given in parenthesis. All patients with chronic liver disease, except those with $_1$-antitrypsin deficiency were found to have serum carotenoids significantly lower than the adult controls (p<0.001 or p<0.05), however all serum levels fell within the normal range for age.

iv)   Serum carotenoid levels in cystic fibrosis.

The serum carotenoid levels obtained from patients with cystic fibrosis were significantly lower (p<0.001) than the adult controls and the unsupplemented liver transplant recipients (p<0.001). Furthermore, serum carotenoid levels declined as age increased (p<0.05). The data has been summarised in Figure 7.4. No correlation between serum carotenoid and vitamin A levels was found in cystic fibrosis.

v)   Serum carotenoid levels in gastrointestinal disease.

The serum carotenoid levels obtained from patients with gastrointestinal disease have been summarised in Figure 7.5. Patients with gastrointestinal disease were found to have significantly lower serum carotenoid levels than the adult controls (p<0.001), but not the unsupplemented liver transplant recipients. No correlation was observed between serum carotenoid and vitamin A levels, in patients with gastrointestinal disease.

(n=9)

2

(n=20)
$

Serum Carotenoid (mg/l)

1

(n=11) (n=51) (n=38)
$ $ $

0

1   2   3   4   5
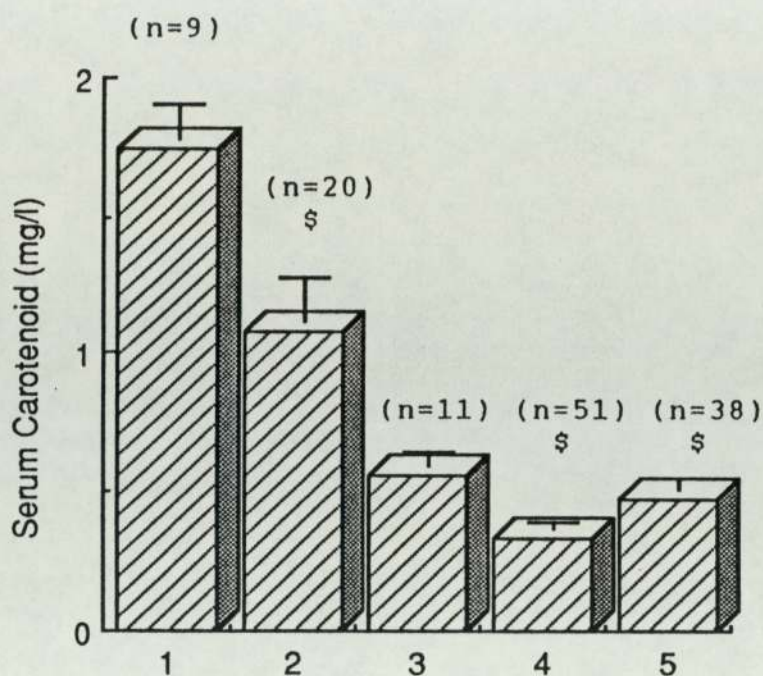
Where:

1   =   Adult controls
2   =   Liver transplant recipients who were not receiving
        vitamin A supplements
3   =   Liver transplant recipients who were receiving
        2,500iu of vitamin A each day
4   =   Patients with cystic fibrosis aged 8-18 years
5   =   Patients with cystic fibrosis aged 1-4 years

* $p < 0.001$
$ $p < 0.05$

Figure 7.4. Histogram representing serum carotenoid levels
in cystic fibrosis. The abscissa shows patients with cystic
fibrosis aged 1-4 years (5) & 8-18 years (4), liver
transplant recipients (unsupplemented (3) and those
receiving 2,500iu of vitamin A each day (2)) and adult
controls (1). The ordinate shows serum carotenoid level
(mg/l). Histobars represent mean serum level of carotenoid,
with central vertical bars giving the SEM. Patients with
cystic fibrosis were found to have serum carotenoid levels
significantly lower than the adult controls and the
unsupplemented liver transplant recipients (p<0.001),
however serum levels remained within the normal range for
age. Furthermore, serum carotenoid levels fell as age
increased in cystic fibrosis (p<0.05).
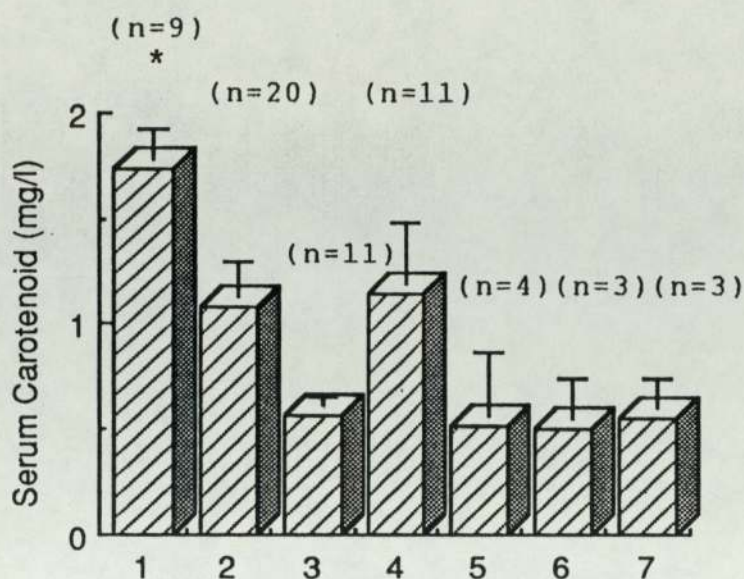
Where:

```
1  =  Adult controls
2  =  Liver transplant recipients who were not receiving
      vitamin A supplements
3  =  Liver transplant recipients who were receiving
      2,500iu of vitamin A each day
4  =  Patients who were failing to thrive
5  =  Patients with short bowel syndrome
6  =  Patients with malabsorption
```

                    *   p < 0.001

Figure 7.5. Histogram representing serum carotenoid levels
in gastrointestinal disease. The abscissa shows patients as
described by diagnosis, whilst the ordinate gives serum
carotenoid level (mg/l). Histobars represent mean serum
carotenoid level, with central vertical bars giving the SEM.
The number of specimens have been given in parenthesis.
Patients with malabsorption were undergoing investigation
for anorexia and weight loss. All patients with
gastrointestinal disease had serum carotenoid levels
significantly lower than the adult controls (p<0.001), but
not the unsupplemented liver transplant recipients.

-193-

vi) Discussion.

In the present study, the adult controls had slightly elevated serum carotenoid levels. However, the mean serum carotenoid level for each group of patients, except the liver transplant recipients who were receiving vitamin A supplements, and the patients with cystic fibrosis fell within the normal range for age (High, 1969).

Serum carotenoid levels reflect immediate dietary intake (WHO, 1982) since carotenoids do not undergo extensive hepatic storage (unlike vitamin A) (Moore, 1957). Therefore, dietary insufficiency or malabsorption rapidly result in a decline in serum carotenoid levels, which is useful in the assessment of malabsorption. As much as two-thirds of the total dietary carotenoids may not possess vitamin A activity, therefore serum carotenoid levels are not particularly useful in the assessment of vitamin A status (Underwood, 1984).

1.   Serum carotenoid levels in adult controls.

The mean serum carotenoid level for the adult controls fell just above the upper limits of the normal range (High, 1969). Similarly serum vitamin A were found to be at the top of the normal range for vitamin A. With the general increasing awareness of diet, carotenoid intake within the population might have increased, resulting in higher serum carotenoid levels than previously quoted.

2.   Serum carotenoid levels in liver transplant recipients.

The lower serum carotenoid levels found in the present
study, in the unsupplemented liver transplant recipients
(6.20±3.04 years, n=4), compared with the adult controls
(25.50±0.71years, n=9) probably result from differences in
diet related to age (O'Neil et al., 1970). Age related
differences in serum carotenoid levels did not appear to be
recognised by all previous investigators (High, 1969).
Although variation in serum carotenoid levels was observed
in the present study, the serum carotenoid levels in the
unsupplemented liver transplant recipients remained within
the normal range for age.

The lower serum carotenoid levels (below the lower
limits of the normal range) in the liver transplant
· recipients supplemented with vitamin A, indicated poor
dietary intake of carotenoids. However, the reason for such
a poor dietary intake or malabsorption was unclear. Although
these patients were younger (1.56±0.25years, n=4) than the
unsupplemented liver transplant recipients, the age
difference did not explain the poor serum carotenoid levels
(High, 1969, O'Neil et al., 1970). One of the patients had
experienced feeding difficulties following the liver
transplant along with a series of infections, therefore a
poor dietary intake of carotenoids was possible. Presumably
serum vitamin A levels in this patient (1.34±0.11μmol/l,
n=11) were maintained from supplemented vitamin A and from

-195-

stores in the donor liver, despite the poor dietary intake. The remaining two patients had perhaps experienced more clinical difficulties post operatively, than the group of unsupplemented transplant recipients, but feeding was not considered a problem at the time of analysis. Furthermore liver function tests were not abnormal in these patients indicating that rejection of the donor liver was not occurring.

Supplemented vitamin A might be absorbed in preference to dietary carotenoids, since absorption of the former has been said to be more complete (El-Goreb et al., 1973). This might result in adequate serum vitamin A levels but lower carotenoid levels. Alternatively, if vitamin A intake remained poor despite vitamin A supplements, a high proportion of dietary carotenoid might have been converted into vitamin A at a mucosal level (Passmore & Eastwood, 1987).

Although there was no overall change in serum carotenoid level following liver transplantation, the mean serum level increased in 3 of the individuals, indicating improved intake or absorption of carotenoids (WHO., 1982). Conversely serum carotenoid levels fell in the remaining 2 patients, one of whom continued to experience feeding difficulties following the liver transplant, as discussed above.

3.   Serum carotenoid levels in chronic liver disease.

No correlation between serum vitamin A and carotenoid levels was found in chronic liver disease, confirming the view that vitamin A status was independent of immediate dietary intake (Underwood, 1984). Although all patients, except those with $alpha_1$-antitrypsin deficiency and glycogen storage disease had serum carotenoid levels less than the adult controls, only those with glycogen storage disease had serum levels which differed from the unsupplemented liver transplant recipients. This indicated that serum carotenoid levels were related to age, presumably as a result of a changing diet, supporting the findings of O'Neil and co-workers (1970). Conversely, High (1969) found that age did not affect serum carotenoid levels.

Elevated serum carotenoid levels were noted in a number of specimens which appeared to be visually lipidaemic, particularly from patients with glycogen storage disease. Although hypercarotenaemia has been reported in association with proprietary baby foods (Stirling et al., 1986), the absence of a deep orange colouration in the serum extract indicated that the higher levels found in patients with glycogen storage disease in the present study resulted from interference in the assay, probably by cholesterol, rather than high carotenoid content (Underwood, 1984).

-197-

4.   Serum carotenoid levels in cystic fibrosis.

Since the patients with cystic fibrosis had significantly lower serum levels than the adult controls and the unsupplemented liver transplant recipients, the lower serum carotenoid levels were likely to result from malabsorption of carotenoid rather than age related differences in dietary intake. The further fall in serum carotenoid levels as age increased in cystic fibrosis, might indicate a reduction in intake with age, or increased malabsorption. However, sufficient vitamin A appeared to be absorbed to maintain serum vitamin A levels.

5.   Serum carotenoid levels in gastrointestinal disease.

Patients with gastrointestinal disease had serum carotenoid levels significantly lower than the adult controls ($p < 0.001$), but not the unsupplemented liver transplant recipients. Therefore the lower serum carotenoid levels were likely to have resulted from changes in dietary intake, related to age.

vii) Summary.

There was no significant correlation between serum carotenoid and vitamin A levels. Disease state, other than cystic fibrosis, did not result in lower serum carotenoid levels when compared with age-matched liver transplant recipients acting as controls in the present study. The

-198-

lower serum carotenoid levels observed in patients when compared with the adult controls probably indicates age related differences in dietary carotenoid intake, however this requires further study, particuarly in view of the low serum carotenoid levels found in the liver transplant recipients supplemented with vitamin A. The low serum carotenoid levels found in patients with cystic fibrosis suggests the presence of malabsorption despite pancreatic supplements (Kelleher, 1987).

CHAPTER 8 : ABSORPTION OF VITAMIN A IN MALABSORPTIVE STATES.
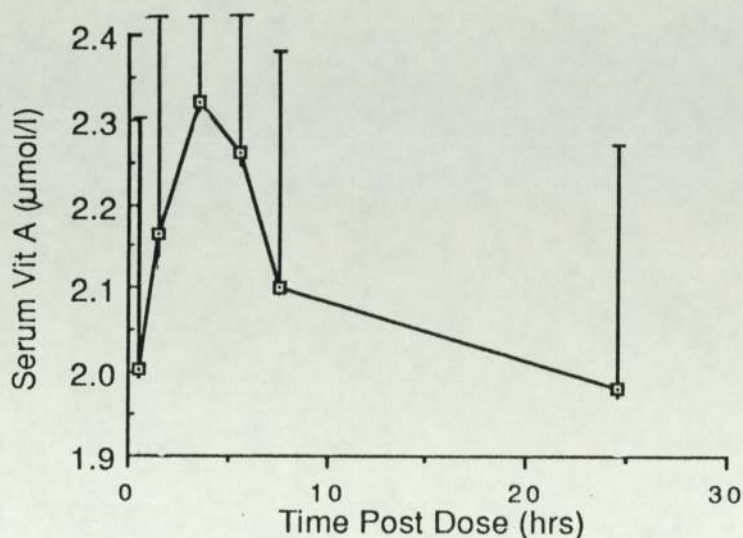
Figure 8.1. Graph representing serum vitamin A levels
following ingestion of 5,000iu of water-miscible vitamin A
in adult controls (n=5). The abscissa shows the time post
dose (hrs), whilst the ordinate gives the serum vitamin A
level (μmol/l). Each point represents the mean of 5
specimens and the vertical bar gives the SEM. The increase
in serum vitamin A levels over the 24 hour period was not
statistically significant, representing only a 19% increase
above the initial serum level.

Figure 8.2. Graphs representing serum vitamin A levels
following ingestion of 5,000iu of water-miscible vitamin A
in individual patients with EHBA (unsuccessful Kasai
operation) (n=3). The abscissa shows time post dose (hrs),
whilst the ordinate shows serum vitamin A level (µmol/l).
Each point represents a single determination of the serum
level. A transient peak serum vitamin A level was observed
4.8±0.6hrs (n=3) following administration of the vitamin A.
This transient peak in serum levels represented an increase
of approximately 47% above the initial serum vitamin A level
(the initial measurement was lost in one patient).

i)   Absorption of vitamin A in adult controls.

Ingestion of 5,000iu (86$\pm$8mg/l) of vitamin A resulted
in an increase in serum vitamin A level of approximately 19%
above the initial serum level in the adult controls (**Figure
8.1**).   However,   this   increase   was   not   statistically
significant.

ii) Absorption of vitamin A in chronic liver disease.

The   absorption   curves obtained when higher   doses   of
vitamin A (996$\pm$126iu/kg) were administered to 9 patients
with chronic liver disease and persistently low serum
vitamin A levels (0.34$\pm$0.05μmol/l,   n=20),   have   been
summarised in Figures 8.2 to 8.4.

Administration   of   5,000iu (Figures 8.2 & 8.3) and
10,000iu of vitamin A (**Figure 8.4**) in patients with chronic
liver disease resulted in a significant increase in serum
vitamin A levels (p<0.05), the transient peak serum level
representing an increase ranging from approximately 47-81%
above the initial serum vitamin A level. The peak serum
vitamin A level was observed 4.9$\pm$1.4 hours (n=7) following
ingestion of vitamin A. Patients with neonatal hepatitis
(serum vitamin A levels increased by approximately 75% above
the initial serum level), alpha$_1$-antitrypsin deficiency, and
idiopathic cirrhosis (approximately 81%) exhibited more
efficient absorption than those with EHBA (approximately
47%). A routine oral daily dose of 5,000iu (917$\pm$225iu/kg) of

-203-

a.   Neonatal hepatitis (n=1).
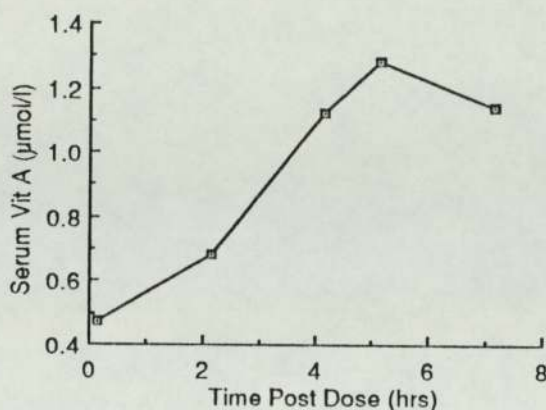
b.   Alpha$_1$-antitrypsin deficiency (n=1).
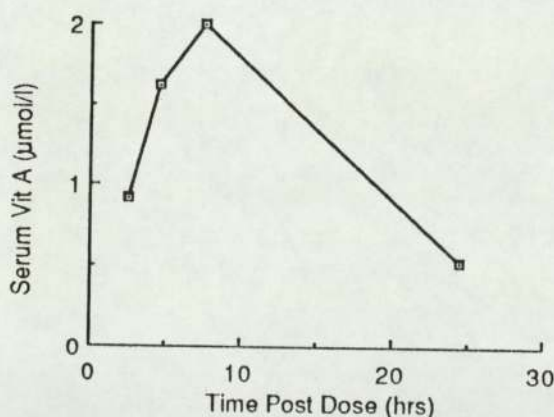
Figure 8.3. Graphs representing serum vitamin A levels
following ingestion of 5,000iu of water-miscible vitamin A
in individual patients with neonatal hepatitis (a.) and α_1-
antitrypsin deficiency (b.). The abscissa shows the time
post dose (hrs), whilst the ordinate shows serum vitamin A
level (µmol/l). Each point represents a single determination
of the serum level.   Serum vitamin A levels rose by
approximately 75% following ingestion of vitamin A, the
transient peak serum vitamin A level occurring after 5.5hrs
(n=2) (the initial measurement was lost in the second
patient).

-204-

a. EHBA following an unsuccessful Kasai procedure.
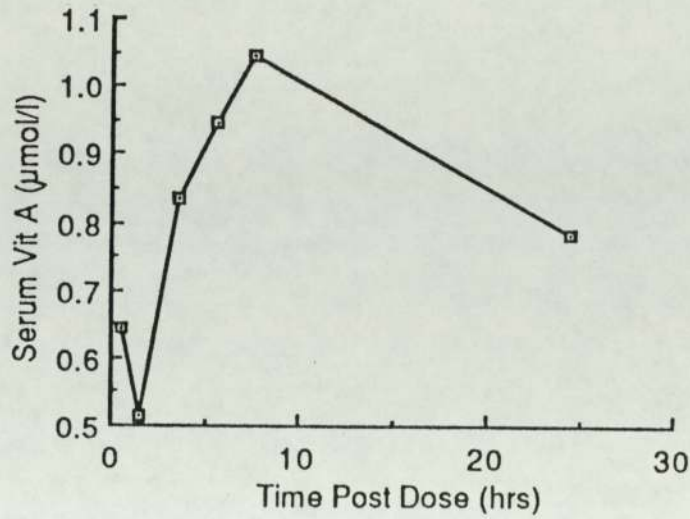
b. Idiopathic cirrhosis (n=1).
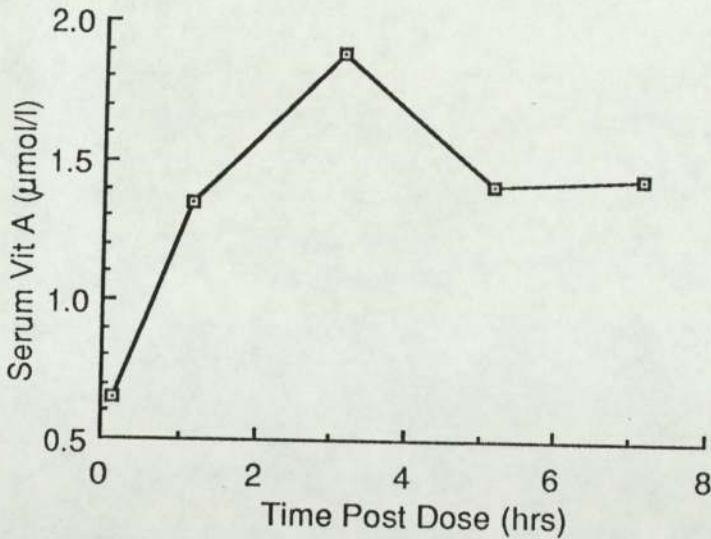
Figure 8.4. Graphs representing serum vitamin A levels following ingestion of 10,000iu of water-miscible vitamin A in individual patients with EHBA (unsuccessful Kasai operation) (a.) and idiopathic cirrhosis (b.). The abscissa shows time post dose (hrs), whilst the ordinate shows serum vitamin A level (µmol/l). Each point represents a single measurement of the serum level. A transient peak in serum vitamin A level was observed 5hrs (n=2) after ingestion of the vitamin A.

vitamin A resulted in the maintenance of serum vitamin A
levels of $1.82\pm0.34\mu$mol/l (n=4) and $1.07\pm0.10\mu$mol/l (n=4) in
the first two patients. However, the patient with
idiopathic cirrhosis did not achieve adequate serum vitamin
A levels ($0.51\pm0.10\mu$mol/l, n=7), despite daily oral doses of
up to 15,000iu (1,250iu/kg) of vitamin A (as Arovit drops
and Ketovite liquid) over a 10 week follow up period.

Of the 4 patients with EHBA, one died, and two received
liver transplants. Post transplant data was available in one
of these patients, who was found to have a mean serum
vitamin A level of 1.59 μmol/l (n=2). Doses of up to
17,500iu (2,573iu/kg) of vitamin A failed to elevate serum
vitamin A levels in the fourth patient which remained
consistently low at $0.42\pm0.06\mu$mol/l (n=9), over a 30 week
follow up period.

iii) Absorption of vitamin A in ultra-short bowel.

The transient peak in serum vitamin A level, following
administration of 10,000iu (400iu/kg) of vitamin A to the
patient with ultra-short bowel represented an increase of
approximately 32% above the initial serum vitamin A level
(Figure 8.5). After initial problems with the stability of
the vitamin A solution (Type 100), a serum vitamin A level
of 2.17μmol/l (n=1) was attained in the patient with ultra-
short bowel, following treatment for 8 weeks with a daily
oral dose of 22,000iu of vitamin A (10,000iu as Ketovite
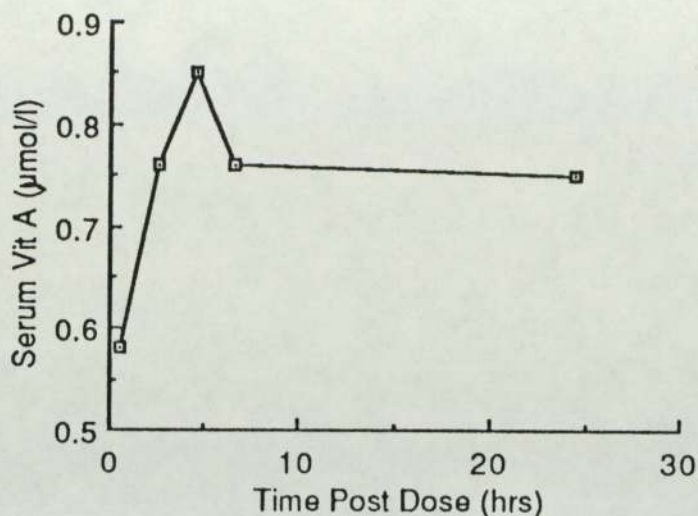
Figure 8.5. Graph representing serum vitamin A levels following ingestion of 10,000iu of water-miscible vitamin A in a patient with ultra-short bowel (14.5cm). The abscissa represents time post dose (hrs), whilst the ordinate shows serum vitamin A level (μmol/l). Each point represents a single determination of the serum level. Serum vitamin A levels rose by 32% above the initial serum level 4hrs after ingestion of vitamin A.
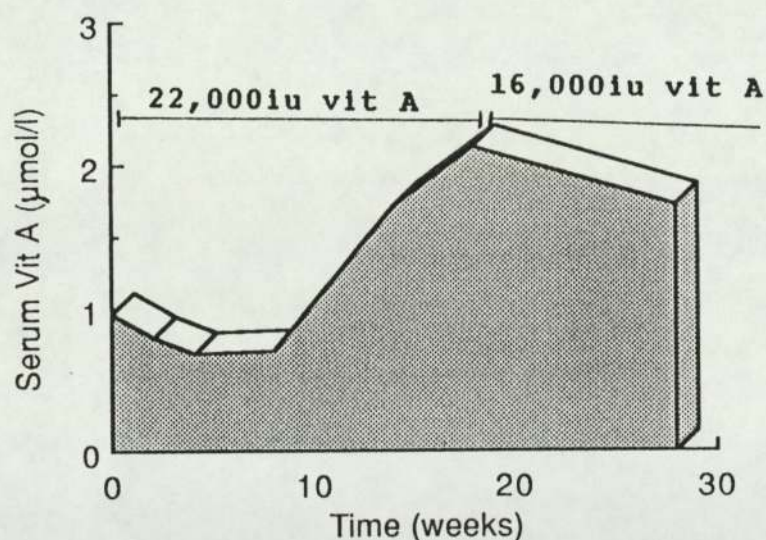
Figure 8.6. Graph representing serum vitamin A levels in the patient with an ultra short bowel, following regular daily vitamin A supplements. The abscissa shows duration of therapy (weeks), whilst the ordinate shows serum vitamin A level (μmol/l). The dose of vitamin A administered has been given above the graph. After initial stability problems a serum vitamin A level of 2.17μmol/l was obtained following treatment with 22,000iu of vitamin A (Type 100 + Ketovite) each day for 18 weeks. The daily dose of vitamin A was then reduced to 16,000iu having converted the vitamin A preparation to the more stable Arovit drops. Serum vitamin A level remained at 1.75$\pm$0.15μmol/l (n=4).

liquid, 12,000iu as Type 100). Dosage was then reduced to 16,000iu of vitamin A, having changed the vitamin A preparation from the Type 100 to the more stable Arovit Drops, and a mean serum vitamin A level of 1.75±0.15μmol/1 (n=4) was maintained (Figure 8.6).

iv) Absorption of vitamin A in abetalipoproteinaemia.

1. Absorption of vitamin A.

Despite administration of 100,000iu (7,692iu/kg) of vitamin A, the maximum increase in serum vitamin A level was only 0.41μmol/1 in the patient with abetalipoproteinaemia. No separate peak in serum vitamin A level was noted, following administration of the vitamin A (Figure 8.7).

2. Absorption in the presence of vitamin E.

A further dose of vitamin A (100,000iu) was administered to the patient with abetalipoproteinaemia, along with 3g (231mg/kg) vitamin E (Vitamin E suspension). No increase in serum vitamin A level was observed over 4 hours of the test, however, 24 hr following ingestion of the test dose, the serum vitamin A level was noted to be increased (0.94μmol/1). This apparent effect of vitamin E, delaying the absorption of vitamin A occurred despite negligible absorption of vitamin E.
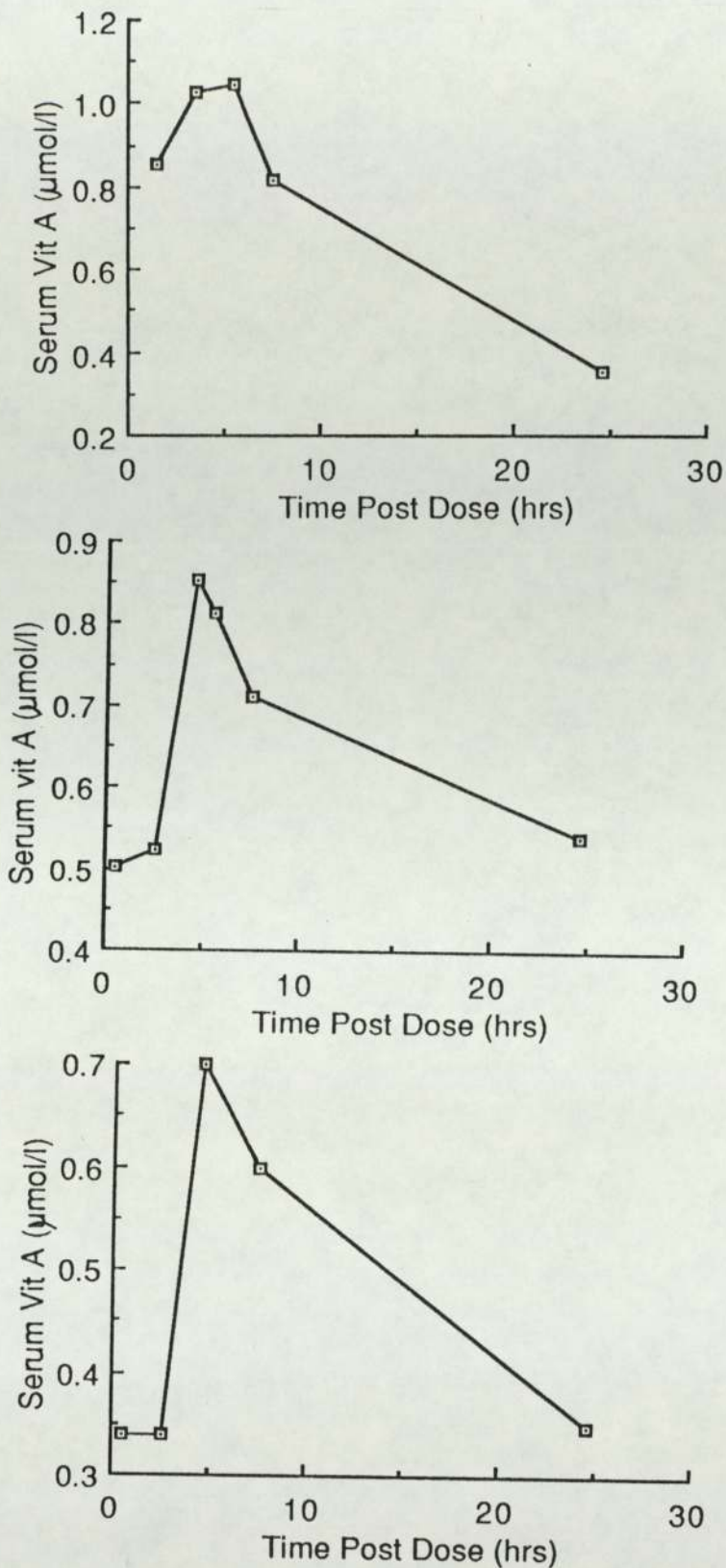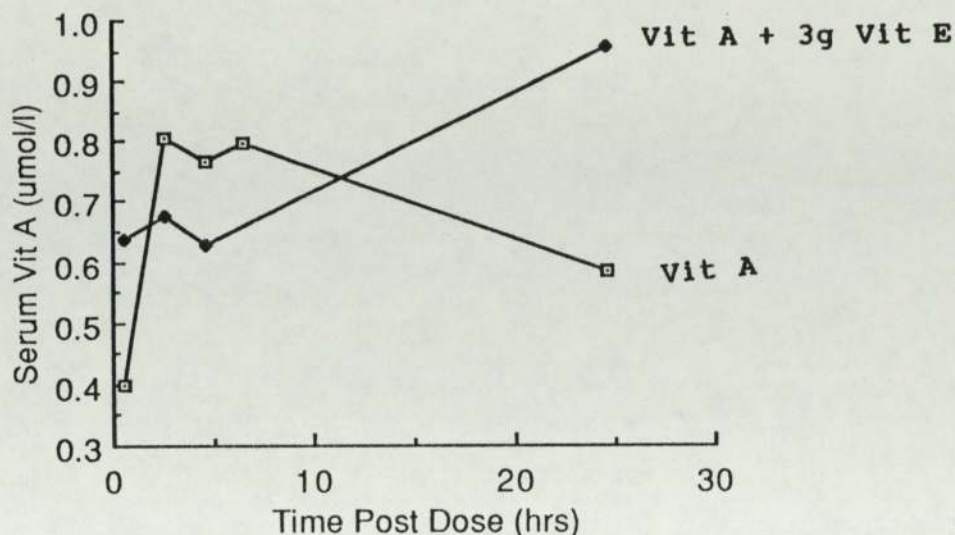
Figure 8.7. Graph representing serum vitamin A levels
following ingestion of 100,000iu of water-miscible vitamin A
(——□—) in a patient with abetalipoproteinaemia. Serum
vitamin A levels were also determined following concurrent
administration of 100,000iu of vitamin A and 3g of vitamin E
(——◆—). The abscissa represents the time post dose (hrs),
whilst the ordinate shows serum vitamin A level (μmol/l).
Each point represents a single determination of the serum
level. Serum vitamin A levels rose following ingestion of
vitamin A, however no separate transient peak serum level
was observed. In the presence of vitamin E, a transient peak
serum vitamin A level was observed 24 hours after ingestion
of the vitamin A. This effect of vitamin E occurred despite
negligible absorption.

-209-

v)   Serum carotenoid levels during absorption of vitamin A.

Fluctuations in serum carotenoid levels were noted during the absorption tests in both the adult controls and patients with chronic liver disease, however these fluctuations were not statistically significant. In contrast, serum carotenoid levels remained undetectable in the patients with an ultra-short bowel and abetalipoproteinaemia.

vi)   Absorption of vitamin E in abetalipoproteinaemia.

The serum levels of vitamin E were virtually undetectable in the patient with abetalipoproteinaemia prior to the absorption test, and remained so following ingestion of 3g vitamin E. Therefore, weekly intramuscular injections of vitamin E (Ephynal) were initiated at a dose of 100mg/week in this patient, and also the patient with short bowel. In the latter patient, regular intramuscular administration of vitamin E resulted in a gradual increase in serum vitamin E level from 4.10µmol/l to 13.81µmol/l over a 5 month period. The dose was then increased to 150mg/week resulting in a serum vitamin E level of 22.50µmol/l following treatment for 3 months. The first patient had a serum vitamin E level of 2.5µmol/l following 2 months therapy however, further results would be required to assess the success of treatment.
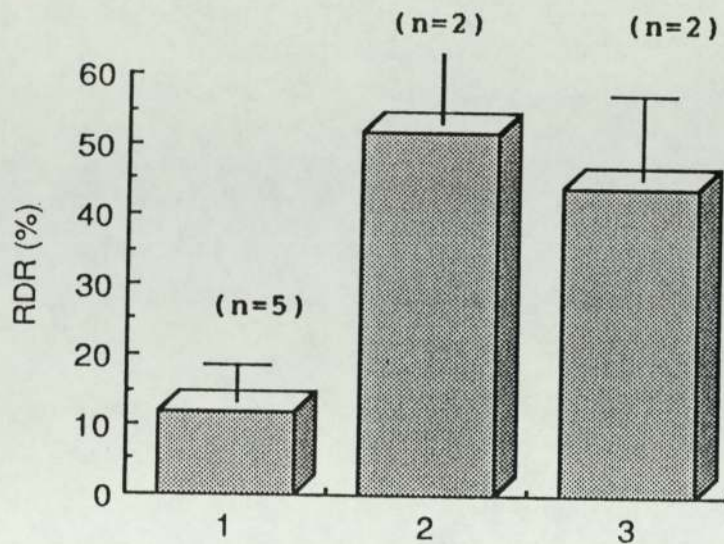
Where:

1   =   Adult controls
2   =   Patients with chronic liver disease following
          ingestion of 5,000iu of vitamin A
3   =   Patients with chronic liver disease following
          ingestion of 10,000iu of vitamin A

Figure 8.8. Histogram representing the relative dose
response (RDR) to a dose of 5,000iu (2) or 10,000iu (3) of
water-miscible vitamin A in chronic liver disease compared
with adult controls (5,000iu of vitamin A) (1). The abscissa
describes patients undergoing the RDR test, whilst the
ordinate shows RDR (%), the percentage increase in serum
vitamin A 5hr after ingestion of the test dose. Histobars
represent mean serum vitamin A levels, with the central
vertical bar giving the SEM for the adult controls. The
number of patients have been given in parentheses. The RDR
was found to be higher in chronic liver disease (44% and 52%
following ingestion of 5,000iu and 10,000iu of vitamin A
respectively), compared with the adult controls (12%).

-211-

vii) Application of the relative dose response test.

In the present study, the principle of the relative dose response test (RDR) (see chapter 11.i.3) was applied to the data obtained from 5 adult controls, 2 patients with chronic liver disease who received 5,000iu of vitamin A, and a further 2 patients who had received 10,000iu of vitamin A (Amedee-Manesme et al., 1987b). The relative dose response was calculated as:

$$\text{RDR} \atop (\%) \qquad \frac{[\text{Vit A}]_5 - [\text{Vit A}]_0}{[\text{Vit A}]_5} \times 100$$

The remaining patients who had undertaken an absorption test were excluded since a 5hr determination of serum vitamin A had not been completed.

The relative dose responses obtained in the present study were found to be < 20% in the adult controls and > 40% in the patients with chronic liver disease (Figure 8.8).

viii) Discussion.

There was no significant increase in serum vitamin A level followed ingestion of 5,000iu (86 ± 8mg/l) of vitamin A in the adult controls, who were not vitamin A deficient. Conversely, serum vitamin A levels did increase following ingestion of a higher dose (996 ± 126iu/kg) of vitamin A in patients with chronic liver disease, ultra-short bowel and

-212-

abetalipoproteinaemia. Despite similar responses to the test
dose of vitamin A, the long term response to regular vitamin
A supplements, in chronic liver disease was varied.

1.   The effect of fasting on absorption of vitamin A.

Patients   were   not   fasted   during   the   vitamin   A
absorption   test,   since   this   was   not   considered   to   be
ethically   justifiable.   There was no significant change   in
serum   carotenoid levels during the test period,   in   either
the controls or the patients in the present study, therefore
intake   of carotenoids appeared to be relatively consistent.
This   supported   the   view that fasting   was   not   necessary
during absorption tests (Kahan, 1969, Kahan, 1970).

2.   Absorption of vitamin A in adult controls.

Studies   with radioactive isotopes demonstrated   almost
complete   absorption   of vitamin A   in   healthy   individuals
(Goodman   et   al.,   1965).   However,   in   the absence   of   a
deficiency state, vitamin A would be transported directly to
the   liver via the lymph (Goodman et al.,   1966) and   stored
(Peterson et al., 1973a), with only a small amount appearing
in the serum (Loerch et al., 1979). Furthermore, the dose of
vitamin   A   administered   to the   adult   controls   was   only
relatively   small,   therefore   a   large   increase   in   serum
vitamin A levels would not be expected.   The 19% increase in
serum   vitamin A level above the initial serum level,   which
was observed in the present study,   was probably entirely in

-213-

the form of vitamin A esters contained in chylomicrons (see chapter 2.i.5). This increase in serum vitamin A levels was higher than the previously reported 10% increase in serum levels (Russell et al., 1978, Goodman et al., 1965), however variation in the maximum increase was expected since differences in gastric emptying time, and rate of clearance, which are proportional to hepatic metabolism and saturation of hepatic RBP stores might occur (Kahan, 1969). Doses similar to those administered to patients with chronic liver disease were not used in the adult controls due to the risk of inducing vitamin A toxicity.

3. Absorption of vitamin A in chronic liver disease.

The much higher percentage increase in serum vitamin A level following ingestion of vitamin A in patients with chronic liver disease, was partially due to the higher dose of vitamin A (996 ± 126 iu/kg) administered. However, these patients were known to have biochemical vitamin A deficiency, and immediate mobilisation of vitamin A, rather than hepatic storage would be expected in order to maintain serum vitamin A levels (Peterson et al., 1973, Goodman et al., 1965). This response might however be limited by malabsorption or defective synthesis and/or release of retinol-binding protein.

Oral maintenance therapy with vitamin A resulted in an increase in serum vitamin A levels in two patients with chronic liver disease, possibly indicating that although malabsorption might occur, absorption of a suitable dose of vitamin A either via the normal route (Goodman & Blaner, 1984) or the portal route (Yueng & Veen-Baigent, 1972) was sufficient to maintain adequate serum vitamin A levels. Alternatively the increase in serum vitamin A levels might be in the form of vitamin A esters (not bound to retinol-binding protein) which are inactive and might cause toxicity (Vahlquist et al., 1978, James et al., 1984).

However some, if not all patients with liver disease might have poor retinol-binding protein status, as a result of defective synthesis and/or release of the protein (Vahlquist et al., 1978). (see chapter 9) Poor retinol-binding protein status might explain why an increase in serum vitamin A levels were not achieved in a number of patients despite regular vitamin A supplements. In such patients the ability to absorb vitamin A might appear similar, or as in the case of the patient with idiopathic cirrhosis, greater than the patients with ultra short bowel and abetalipoproteinaemia who achieved adequate serum vitamin A levels. Fulton and co-workers (1982) demonstrated a similar response in cystic fibrosis; an initial increase in serum vitamin A levels was not sustained, and it was suggested that hepatic retinol-binding protein release was

impaired. However the possibility of poor compliance was accepted.

A further consideration was that liver disease might well deteriorate; two patients have since received liver transplants, and one has died, whereas the condition of the patients with ultra-short bowel and abetalipoproteinaemia remained stable. The capacity of patients with liver disease to assimilate vitamin A might therefore alter over a relatively short period of time, if either retinol-binding protein status or degree of malabsorption changed as a result of deteriorating liver function.

4.   Absorption of vitamin A in abetalipoproteinaemia and short bowel syndrome.

Despite the apparently poor ability of the patients with abetalipoproteinaemia and ultra-short bowel to absorb vitamin A, maintenance therapy with 16,000iu and 100,000iu of vitamin A respectively, resulted in attainment of adequate serum vitamin A levels over an 11 month period. In abetalipoproteinaemia chylomicrons are absent (Bishara et al., 1982), and therefore vitamin A, along with the other fat-soluble vitamins must be absorbed by an alternative pathway, presumably the portal route (Yueng & Veen-Baigent, 1972, Murray & Grice, 1961). However, synthesis of retinol-binding protein is independent of apoprotein B and is therefore not affected by abetalipoproteinaemia (Bieri et

-216-

al., 1984). The utilisation of a different route of absorption might explain why no separate transient peak in serum vitamin A levels was observed in this patient, however Yeung and Veen-Baigent (1972) demonstrated a peak serum level, although this occurred earlier in the absorption test.

Both patients were expected to have normal retinol-binding protein status, therefore low serum vitamin A levels were likely to result from malabsorption of vitamin A (Smith & Lindenbaum, 1974, Vahlquist et al., 1978, Bieri et al., 1984). The findings in the present study indicated that malabsorption could be overcome with a suitable oral dose, an accumulative effect being observed with regular dosing.

5.   The absorption of vitamin A in the presence of vitamin E.

In the present study, concurrent administration of vitamin E appeared to delay the absorption of vitamin A in the patient with abetalipoproteinaemia, despite negligible absorption of the former vitamin. Further investigation is required to confirm the nature of this interaction, which Willett and co-workers (1983) suggested resulted from competition for micellar solubilisation. There were no previous reports of delayed, as opposed to reduced absorption of vitamin A (Bieri et al., 1984). Conversely, moderate doses of vitamin E have been shown to enhance the

absorption of vitamin A (Kusin et al., 1974), however this effect was offset by increased urinary losses.

6. The relative dose response test.

The relative dose responses obtained in the present study indicated the presence of adequate hepatic vitamin A stores in the adult controls, and depleted hepatic stores in the patients with chronic liver disease. The hepatic vitamin A stores could not be quantified in these patients, since different doses of vitamin A were administered in the present study (see chapter 11.i.3) (Champos et al., 1987).

ix) Summary.

Although absorption of vitamin A appeared to be poor in patients with chronic liver disease, ultra-short bowel and abetalipoproteinaemia in the present study, increases in serum vitamin A levels of between 32-81% above the initial serum level were observed, approximately 5 hours after ingestion of vitamin A. The increase in serum vitamin A levels did not appear to be dose dependent, however further investigation is required to confirm these findings since individuals may have widely differing abilities to absorb vitamin A. Despite poor absorption of vitamin A, adequate serum levels were attained with regular vitamin A supplements in patients with ultra-short bowel, abetalipoproteinaemia, neonatal hepatitis and $alpha_1$-antitrypsin deficiency. These findings might indicate that

the primary cause of vitamin A deficiency in these patients was malabsorption, which could be overcome if a suitable dose of vitamin A was administered. Alternatively, any increase in serum vitamin A levels might be due to an increase in serum concentration of vitamin A esters in the absence of retinol-binding protein, and this might ultimately result in toxicity (Vahlquist et al., 1978). Conversely, patients with EHBA and cirrhosis did not respond to regular vitamin A supplements despite having demonstrated a similar ability to absorb vitamin A, indicating that other factors might be involved in the development of low serum vitamin A levels. Any initial increase in serum vitamin A levels might be due to an increase in the serum concentration of vitamin A esters. If higher doses of vitamin A were employed in these patients, the serum concentration of vitamin A esters might continue to increase, ultimately resulting in toxicity (Vahlquist et al., 1978).

CHAPTER 9 : SERUM TOTAL RETINOL-BINDING PROTEIN LEVELS IN
MALABSORPTIVE STATES.

i)   Intra-assay and inter-assay variaition

        The   mean inter-assay variation (V) for the ELISA assay
of serum total RBP was calculated as 14% (range 0.5 to 33%)
for 19 specimens, assayed on two separate occasions.   The
inter-assay variation was also calculated for each dilution
of the above specimens and the results have been summarised
in Table 9.1. The overall mean inter-assay variation,
excluding that of the 1 in 16000 dilution where numbers were
too small was 15%.

Dilution of Specimen	No of Pairs	Mean V (%)	Range V (%)
1 in 1000	8	12	0.3-32
1 in 2000	10	15	0.8-31
1 in 4000	8	18	5-27
1 in 8000	4	13	2-24
1 in 16000	2	9	2-15

Table 9.1.   The inter-assay variation (V), calculated for
each dilution of specimens assayed on 2 separate occasions
using the ELISA assay for serum total RBP.

        The inter-assay variation (44%) was also determined for
dilutions of the standard serum preparation used in 10
assays. The results have been summarised in Table 9.2.

-221-

Dilution of Serum Standard	(n)	No of Assays	Absorbance Mean	(SEM)	V (%)
1 in 2000	39	10	0.799	(0.03)	24
1 in 4000	40	10	0.679	(0.04)	25
1 in 8000	40	10	0.485	(0.03)	33
1 in 16000	40	10	0.270	(0.02)	42
1 in 32000	35	10	0.113	(0.01)	69
1 in 64000	27	10	0.065	(0.01)	68

Table 9.2. Inter-assay variation (V) for the serum standard assayed on 10 separate occasions using the ELISA assay for serum total RBP.

Dilution of Serum Standard	(n)	Absorbance Mean	(SEM)	V (%)
1 in 2000	5	0.983	(0.06)	14
1 in 4000	5	0.859	(0.06)	15
1 in 8000	5	0.667	(0.05)	15
1 in 16000	5	0.368	(0.03)	17
1 in 32000	4	0.134	(0.02)	20
1 in 64000	5	0.065	(0.01)	22

Table 9.3. Intra-assay variation for the serum standard (n=5) used in the ELISA assay for serum total RBP.

The intra-assay variation (17%) was calculated for each dilution of the serum standard, and the results have been summarised in Table 9.3.

ii)   Serum total retinol-binding protein levels in healthy

controls.

The serum total RBP levels in healthy adult controls (87±18mg/l, n=5), fell above the upper limits of the normal range quoted by Bosin and Monji (1983) (30-60mg/l), corresponding to serum vitamin A levels (1.99±0.30µmol/l, n=5) at the top of the normal range for vitamin A.

iii) Serum total retinol-binding protein levels in liver

transplant recipients.

Serum total RBP levels were significantly lower in the liver transplant recipients (45±7mg/l, n=11) than the normal controls (p<0.05) (Figure 9.2). However, the mean serum total RBP level of the former fell within the accepted normal range of 30-60mg/l (Bosin & Monji, 1983). Despite a significant fall in serum vitamin A level during episodes of rejection of the donor liver (p<0.05), the serum total RBP levels did not change in the 2 patients for whom data was available.

Since a significant difference in serum total RBP levels was noted between the liver transplant recipients and the adult controls, the serum total RBP levels obtained from
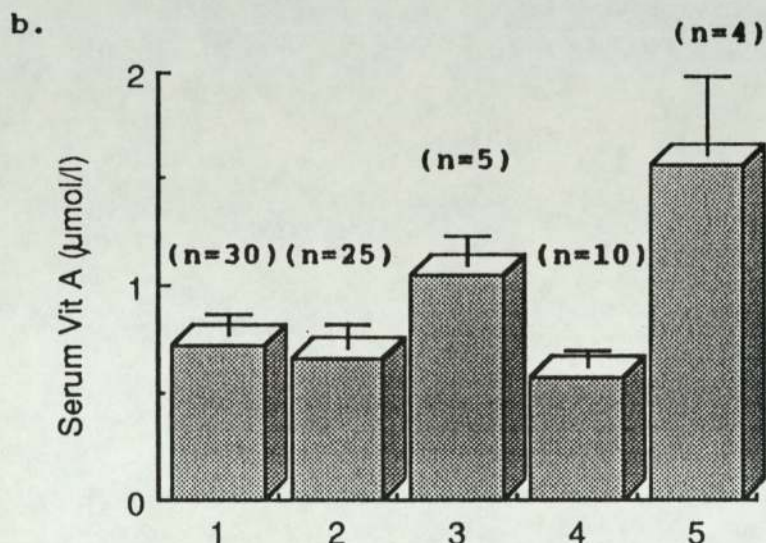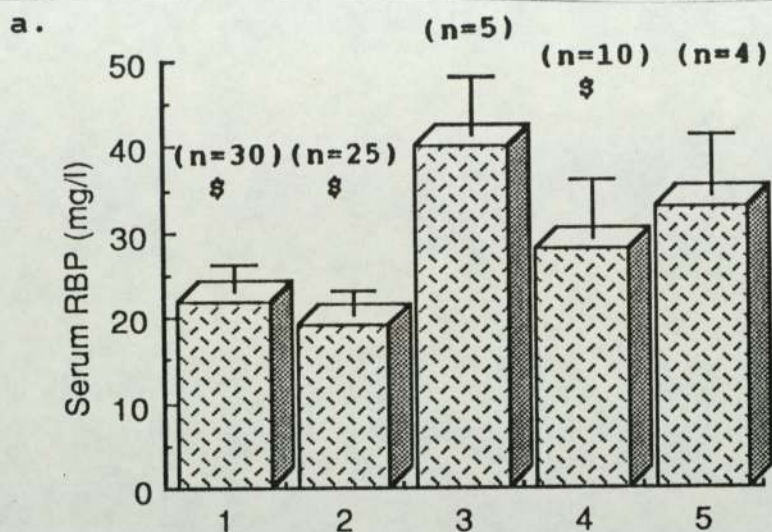
Figure 9.1. Histograms representing serum total RBP (a.) and corresponding serum vitamin A levels (b.) in chronic liver disease. The abscissa shows patients as described by diagnosis, and distinguishes between those patients who have had a successful Kasai operation in EHBA (3) from those in whom the operation was unsuccessful (2). The ordinate shows serum total RBP level (mg/l) (a.) or serum vitamin A level (μmol/l). Histobars represent mean serum level, with central vertical bars giving the SEM, and the number of specimens has been given in parenthesis. Serum total RBP levels were found to be significantly lower in patients with EHBA and cirrhosis ($p<0.05$), but not neonatal hepatitis when compared with liver transplant recipients. Failure of the Kasai operation in EHBA resulted in a further fall in total RBP level ($p<0.05$). A correlation between serum total RBP and vitamin A levels was noted ($p<0.05$).

subsequent groups of patients were compared with both sets of data.

iv) Serum total retinol-binding protein levels in chronic liver disease.

The serum total RBP levels found in patients with chronic liver disease have been summarised in Figure 9.1. Serum total RBP levels in EHBA (1.69±10.34 years, n=16) and cirrhosis (1.70±0.16 years, n=6) were significantly lower than both the adult controls (p<0.001) and liver transplant recipients (p<0.05). However, these serum total RBP levels remained just within the lower limits of the normal range for age, although serum vitamin A levels were low (Vahlquist et al., 1975). Failure of the Kasai operation in patients with EHBA was associated with a further decrease in serum total RBP levels (p<0.05). Patients with neonatal hepatitis and alpha$_1$-antitrypsin deficiency were found to have significantly lower serum total RBP levels that the adult controls (P<0.05) but not the liver transplant recipients.

The individual patients, who could not be included in statistical analysis since the number in each group was too small have been considered separately (Table 9.4). The patients with Wilson's Disease, hypercarotenaemia, and malabsorption appeared to have adequate serum levels of both total RBP and vitamin A. However serum total RBP and vitamin A levels were found to be low in the patients with

-225-

sclerosing cholangitis, congenital hepatic fibrosis and
primary biliary cirrhosis (PBC) (adults). Likewise the
subject with abetalipoproteinaemia was also found to have a
low serum total RBP level, despite a normal serum vitamin A
level.

Diagnosis	(n)	Mean serum total RBP (mg/l)	Mean serum Vit A (µmol/l)
Wilson's Disease	(1)	66	1.58
Sclerosing cholangitis	(1)	27	0.66
Congenital hepatic fibrosis	(1)	35	1.04
Abetalipoproteinaemia	(1)	23	1.10
Primary Biliary Cirrhosis	(2)	27	0.68

Table 9.4. Serum total RBP levels in remaining patients with
chronic liver disease (n=6).

v)   Serum total retinol-binding protein levels in cystic
     fibrosis.

     Cystic fibrosis did not appear to affect serum total
RBP levels, since there was no significant difference
between the serum total RBP levels found in the patients
with cystic fibrosis, the adult controls or the liver
transplant recipients (Figure 9.2). These serum total RBP
levels corresponded to a serum vitamin A level of
1.46 ± 0.30umol/l (n=10).

-226-
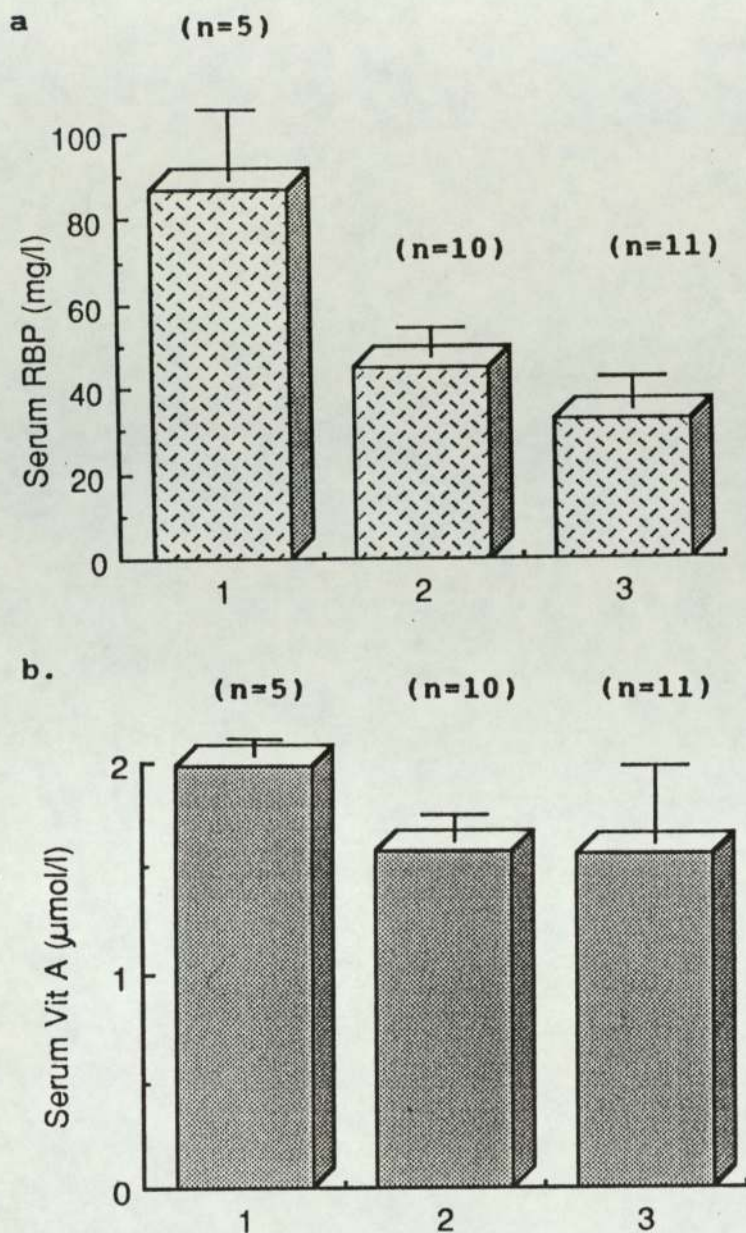
Where:

1   =   Adult controls
2   =   Patients with cystic fibrosis
3   =   Liver transplant recipients

Figure 9.2. Histograms representing serum RBP levels and corresponding serum vitamin A levels in cystic fibrosis. The abscissa shows patients with cystic fibrosis (2), compared with adult controls (1) and the liver transplant recipients (3), whilst the ordinate shows serum RBP level (mg/l) (a.) or serum vitamin A level (μmol/l) (b.). Histobars represent mean serum level, with central vertical bars giving the SEM. The number of specimens have been given in parenthesis. Serum RBP levels in cystic fibrosis were not significantly different to serum vitamin A level from the adult controls or the liver transplant recipients. A correlation between serum RBP and vitamin A levels was noted ($p < 0.05$).

vi) The relationship between serum total retinol-binding
protein and vitamin A levels.

A significant correlation between serum total RBP and
vitamin A levels was found in the adult controls (p<0.05),
liver transplant recipients (p<0.05), and patients with EHBA
(p<0.001) or cystic fibrosis (p<0.05). The remaining groups
of patients were not included in the analysis, since the
numbers were too small.

vii) The effect of ingestion of vitamin A on serum
total retinol-binding protein levels.

Ingestion of 5,000iu (86 ± 8iu/kg) of vitamin A did not
result in elevation of serum total RBP or vitamin A levels
in the adult controls (n=5). Conversely, ingestion of
5,000iu or 10,000iu of vitamin A (996 ± 126iu/kg) did result
in a significant increase in serum vitamin A levels
(p<0.05), but not serum total RBP levels in the 2 patients
with chronic liver disease, for whom serum total RBP levels
were available.

viii) The response of serum total retinol-binding protein
levels to changes in serum vitamin A levels.

The serum total RBP and corresponding vitamin A levels
were determined in 11 patients at weekly intervals, to
investigate how closely the two parameters were associated,
within an individual. The details of the individuals have
been given in Table 9.5.

Diagnosis	Subject Number	Serum Total RBP Level (mg/l)	Serum Vit A Level (µmol/l)	Dose* of Vit A (iu/day)
Liver transplant recipients	1	32	1.40	————
		64	2.22	
	2	19	1.50	————
		18	0.70	
		43	1.49	
	3	52	2.26	————
		56	1.32	
	4	36	1.34	2,500
		26	0.77	
	5	73	0.82	2,500
		95	1.95	
Idiopathic cirrhosis	6	9	0.61	2,500
		37	0.37	5,000
		5	0.18	5,000
		40	0.90	15,000
EHBA	7	8	0.28	7,500
		6	0.36	
		6	0.35	
		3	0.67	
		18	0.53	17,500
	8	17	1.22	5,000
		20	1.10	
		16	0.53	
		17	0.72	
	9	9	0.20	15,000
		22	0.10	25,000
		13	0.20	
	10	11	0.31	2,500
		12	0.35	5,000
	11	10	0.58	2,500
		68	1.57	7,500

* Dose of pre-formed vitamin A, administered in addition to any dietary carotenoid or vitamin A.

Table 9.5. Serum total RBP & vitamin A levels in individual patients (n=11).

Three of the liver transplant recipients (aged 1, 8, 14 years) were receiving no additional vitamin A supplements, whilst two (aged 1 & 2 years old) received 2,500iu of vitamin A each day (as Ketovite liquid). All patients maintained adequate serum vitamin A levels, although fluctuation was noted, as expected in a healthy individual (Underwood, 1984). A particular drop in serum vitamin A levels was observed in patients 2 and 4 (Table 9.4) in association with severe episodes of diarrhoea. A concurrent decline in serum total RBP level was also observed.

The serum total RBP levels in subject 6 with idiopathic cirrhosis (Table 9.5) rose from 9 to 37mg/l, following a daily dose increase from 2,500iu to 5,000iu of vitamin A, despite a fall in serum vitamin A level; the serum total RBP level then fell to 5mg/l. An increase in the daily dose of vitamin A from 7,500iu to 17,500iu of was associated with an increase in serum total RBP but not vitamin A levels in patients 7 and 9, in a similar way to the first increase noted in patient 6. Conversely, serum total RBP did not fall in association with a decline in vitamin A in subject 8. An increased vitamin A intake was not associated with an increase in serum vitamin A or total RBP levels in subject 10, but the converse was true in subject 11.

If the patients were considered as a single group serum vitamin A and total RBP levels remained correlated (p<0.001), however when patients 6-9 were considered as

-230-

individuals there was no correlation between the 2 parameters.

ix) Discussion.

Whereas all patients were found to have serum total RBP levels less than those found in the healthy adult controls, only chronic liver disease resulted in serum total RBP levels lower than those found in the liver transplant recipients.

1.   Assay Variation.

The mean inter-assay variation for each dilution of specimens assayed by the ELISA assay of serum total RBP (mean of 14%) was similar to that previously reported by Dawson (1986). However, the mean inter-assay variation of dilutions of the standard serum preparation used in 10 assays (mean of 44%) was very disappointing. In addition the mean intra-assay variation (17%) was also higher than previously reported (Dawson, 1986). Operator inexperience might partially explain the high degree of variability, however in addition, the serum may have been affected by prolonged storage at -20°C over a 3 year period. These findings emphasise the need to perform a calibration for each microtitre plate used in the ELISA assay, since inter-assay variation for specimens could then be reduced to an acceptable level (mean of 14%).

2.   Serum   total retinol-binding levels   in   healthy
      individuals.

The serum total RBP levels obtained from the adult
controls were found to be above the upper limits of the
normal range (Bosin & Monji, 1983), and were associated with
serum vitamin A levels at the top of the normal range for
vitamin A. Serum levels in children between the ages of 2-10
years were expected to be approximately 60% of the adult
levels   (18-36mg/l),   increasing   during   puberty   and
adolescence to reach adult levels (Vahlquist et al., 1975).

3.   Serum total retinol-binding protein levels in liver
      transplant recipients.

The mean serum total RBP level obtained from liver
transplant recipients in the present study, was in fact
greater than the mean level (21 ± 6mg/l) determined by
Vahlquist and co-workers (1975) in healthy children of
similar age, however the mean serum total RBP level attained
in the adult controls in the present study was also higher
than previously reported (Bosin & Monji, 1983). There was no
evidence to suggest that the liver transplant recipients
would have reduced serum total RBP levels since all had
normal serum bilirubin and albumin levels at the time of
analysis (see chapter 6.xi.1), therfore the patients were
used as age matched controls for the present study.

The serum total RBP levels in the liver transplant recipients were 52% of those found in the adult controls in the present study, indicating that although the results appeared to be higher than previously reported, the expected age related differences in serum total RBP level were still present. The reason for the higher than expected results was unclear, however prolonged storage of the serum standard for total RBP might have resulted in an apparent increase in total RBP concentration. Since each specimen was compared with the standard, a correspondingly high serum total RBP level would be expected. Further evidence for this hypothesis was obtained from the higher than expected serum total RBP levels found in chronic liver disease (see below).

4.   Serum total retinol-binding protein levels in chronic liver disease.

Although the serum total RBP levels found in chronic liver disease fell just within the lower limits of the previously reported normal range (Vahlquist et al., 1975), the serum total RBP levels were only 30% of those found in the adult controls in the present study. As previously discussed, a healthy child would be expected to have serum total RBP levels 60% of those found in an adult. Furthermore the 39% fall in serum total RBP levels found in patients with EHBA in the present study, confirmed the findings of Amedee-Manesme and co-workers (1985), who demonstrated a 29% fall in levels in patients with cholestasis. These findings

-233-

confirmed the hypothesis that the serum total RBP levels obtained in the present study were higher than expeceted, but that age and disease related differences in serum total RBP levels were similar to those previously reported.

Since lower serum total RBP levels were found in chronic liver disease when compared with the liver transplant recipients, reduced synthesis or release of RBP along with malabsorption of vitamin A was thought to have occurred in these patients. When considered as separate groups, the lowest serum total RBP levels were found in patients with the most severe liver disease, EHBA where the Kasai operation had been unsuccessful, or cirrhosis. Conversely, serum total RBP levels similar to the liver transplant recipients were found in patients with EHBA where the Kasai operation had been successful. Although the long term prognosis for such patients might be poor, cirrhosis was not progressive at the time of analysis.

Patients with neonatal hepatitis were found to have serum total RBP levels similar to those found in the liver transplant recipients. These findings were anticipated since these patients had no clinical evidence of significant cirrhosis, and jaundice was either absent or resolving. The patients were however all young children (0.30 ± 0.13 years, n=4) and would therefore be expected to have serum total RBP levels at the lower end of the normal range.

In the present study, the results indicated that two factors might be involved in the pathogenesis of defective synthesis and/or release of total RBP. The lowest serum total RBP levels were found in patients with both cholestasis and progressive cirrhosis. Whilst extensive cirrhosis could perceivably affect synthesis or release of total RBP, the role of cholestasis remained unclear. However Skrede and co-workers (1975) suggested hepatic protein synthesis could be affected by bile retention. Although transthyretin was found to be least affected by biliary obstruction, the effect on total RBP was not discussed. Furthermore, biliary obstruction would be expected to reduce vitamin A absorption and therefore, the amount of vitamin A available for complexing with RBP. This would limit the release of holo-RBP (Muto et al., 1972, Smith et al., 1973a). Further work is required to investigate the aetiology of the defect in RBP status, and the relative influence of cholestasis and cirrhosis.

Although there were numerous reports of reduced serum total RBP levels in liver disease, it was not always clear whether this was caused by poor vitamin A intake resulting from malabsorption (Nyberg et al., 1988), or whether RBP synthesis and/or release was impaired (Smith & Goodman, 1979, Vahlquist et al., 1978). However, studies in which serum total RBP levels were not increased when vitamin A was administered by the intramuscular route, avoiding

malabsorption indicated that RBP synthesis and/or release was impaired, as did studies where dark adaptation was not improved by administration of vitamin A. (Russell et al., 1978, Vahlquist et al., 1978).

5.   Serum total retinol-binding protein levels in cystic fibrosis.

In the present study, patients with cystic fibrosis were found to have higher serum total RBP levels than previously reported (Smith et al., 1972), corresponding with adequate serum vitamin A levels. This did not appear to be related to the general increase in serum total RBP levels observed in the present study (see above), since serum total RBP levels in cystic fibrosis were not significantly different from those serum levels found in the adult controls or the liver transplant recipients.

Although serum total RBP levels did not appear to be reduced in cystic fibrosis in the present study, the RBP might have a reduced affinity for vitamin A (Large et al., 1980, Amedee-Manesme et al., 1985). Alternatively, the proportion of apo-RBP might be increased (Peterson, 1971). The normal serum vitamin A levels found in these these patients might indicate that vitamin A transport was adequate, however there might be an increased concentration of unbound vitamin A ester present in serum. These findings contradicted previous studies however, if defective RBP

synthesis and/or release was a secondary feature of cystic fibrosis the younger age of the patients in the present study (4.59 ± 1.99 years, n=10), might explain why serum total RBP levels appeared adequate (Smith et al., 1972).

6.   The effect of ingestion of vitamin A on serum total retinol-binding protein levels.

In the adult controls the serum total RBP and vitamin A levels did not change significantly following ingestion of 5,000iu of vitamin A. Since these individuals were not vitamin A deficient, immediate mobilisation of ingested vitamin A would not be necessary, and hepatic storage would have occurred (Peterson et al., 1973). Any increase in serum vitamin A would be expected to result from vitamin A esters passing to the liver within chylomicron remnants (Goodman et al., 1965), accounting for less than 20% of the total serum vitamin A level (Russell et al., 1978). Since this small increase in vitamin A esters was independent of RBP, no increase in the serum levels of the latter would be anticipated.

Serum vitamin A levels increased in the two patients with chronic liver disease (for whom serum total RBP levels were available), following ingestion of 5,000iu or 10,000iu of vitamin A, however there was no change in serum total RBP level. An increase in serum total RBP would be expected since patients were known to have biochemical vitamin A

deficiency, therefore vitamin A would be immediately mobilised from the liver, appearing in serum complexed with RBP (Peterson et al., 1973). The lack of response in serum RBP level might have resulted from defective synthesis and/or release of RBP, as has been suggested in chronic liver disease (Vahlquist et al., 1978). Increases in serum total RBP have been demonstrated in rats (Muto & Goodman, 1972) and humans following ingestion of vitamin A (Smith et al 1973a); a rapid increase in serum total RBP level was observed over 5 hours in rats fed a vitamin A depleted diet, followed by oral repletion of vitamin A. However, in these animal studies RBP status was not compromised.

In the 2 patients with chronic liver disease, serum vitamin A levels were increased by > 50% above the initial serum level following ingestion of vitamin A. Since previous investigators have suggested that vitamin A esters contained in chylomicrons accounted for only 10% of the total serum vitamin A (Goodman et al., 1965), it was unclear how vitamin A was appearing in the serum without an increase in serum total RBP level. Serum total RBP and vitamin A are normally associated in a molar ratio of approximately 1:1, with less than 10% (3-4mg/l) of the total RBP being apo-RBP (ie. unbound to vitamin A) (Peterson, 1971). The apo-RBP occurs in serum following transport of the vitamin A to the tissues, and is rapidly catabolised in the kidney. Vitamin A might utilise less specific, low affinity binding sites on

-238-

beta-globulins or beta-lipoprotein in the absence of RBP, or following saturation of RBP (Baker et al., 1967) which occurs at 20-30µg/g (Olson, 1982). Alternatively the proportion of vitamin A esters in serum may have increased (James et al., 1984); this might explain why serum vitamin A levels increased without a concurrent increase in serum total RBP levels and requires further investigation because of the implications for vitamin A toxicity (see chapter 2.vi).

The findings in the present study were in no way conclusive. Further studies are required to establish a trend(s) in liver disease, and also to determine the effect of high doses of vitamin A on total RBP levels in normal controls. Defective RBP synthesis or release might explain why patients who showed a similar ability to absorb vitamin A did not all attain adequate serum vitamin A levels with regular supplements (see chapter 8.ix.3.).

If vitamin A deficiency was simply a problem of malabsorption, supplementation with large doses of vitamin A might overcome the deficiency. If RBP was also deficient, there was a risk that large doses of vitamin A would be absorbed, but would circulate in serum as unbound vitamin A esters, ultimately causing toxicity (Vahlquist et al., 1982).

7. The response of serum total retinol-binding protein levels to changes in serum vitamin A levels.

Fluctuation of serum vitamin A levels in a healthy individual has been well established (Underwood, 1978). The response of serum total RBP levels to such fluctuations has not been so extensively investigated. Since RBP forms a 1:1 complex with vitamin A similar fluctuations would be expected, however Muto and co-workers (1972) demonstrated a time lag of approximately 3 days between the decline in serum vitamin A levels induced in rats by excluding vitamin A from the diet, and the decline in serum total RBP levels.

A correlation between serum total RBP and vitamin A levels was confirmed in adult controls, cystic fibrosis and EHBA in the present study. Furthermore, diarrhoea in liver transplant recipients was associated with a decline in both serum vitamin A and total RBP levels. Diarrhoea presumably affected the absorption of vitamin A, thereby reducing serum levels. The synthesis or release of RBP would not be affected, and the observed decline in serum total RBP probably resulted from a reduction in vitamin A availability (Vahlquist et al., 1978). These findings suggested that the serum total RBP levels closely followed the pattern of fluctuation observed in the serum vitamin A levels.

However, when serum vitamin A levels fell in association with episodes of rejection of the donor liver in

-240-

the liver transplant recipients, serum total RBP levels did not fall. Likewise, the pattern of fluctuation was not found to be consistent in patients with chronic liver disease. An increase in serum total RBP was noted in one patient with idiopathic cirrhosis (patient 6, Table 9.5), following a dosage increase from 2,500iu to 5,000iu of vitamin A, despite a fall in vitamin A level. The serum total RBP level then fell again. Vitamin A might have stimulated RBP release at a gastrointestinal mucosal level, but then malabsorption prevented a concurrent increase in vitamin A level. However, several investigators suggested that stimulation of RBP by vitamin A occurs within the hepatocyte (Smith et al., 1978, Muto et al., 1972, Peterson et al., 1973). The interim high serum level of total RBP might have resulted from experimental error, however if synthesis but not release of RBP was defective, then an initial increase in serum total RBP might occur, but this would not be maintained as hepatic synthesis was reduced (Vahlquist et al., 1978).

As the dose of vitamin A was increased to 15,000iu in the above patient, the serum total RBP level again rose, this time accompanied by an increase in vitamin A level. This increase was however, no greater than the previous one, indicating that the proportion of apo-RBP (not bound to vitamin A) might have been increased in the previous determination (Peterson, 1971). Large and co-workers (1980) demonstrated that apo-RBP still circulated in vitamin A

deficiency, but that it was denatured, and therefore could not bind with vitamin A. This finding was confirmed in children with cholestasis, in whom serum total RBP was only 56% saturated with vitamin A, compared with 81% saturation in healthy children (Amedee-Manesme et al., 1985). Depletion studies in rats did not support these findings, however dietary deficiency was induced in these animal studies, in which malabsorption was not present (Muto & Goodman, 1972).

The loss of correlation between serum total RBP and vitamin A levels in patients 6-9 (Table 9.3) indicated that the molar ratio of RBP to vitamin A might be altered in these individuals, possibly as a result of altered affinity of vitamin A for RBP (Large et al., 1980, Amedee-Manesme et al., 1985), utilisation of non-specific binding sites on other proteins (Baker et al., 1967), or circulation of increased quantities of vitamin A esters (James et al., 1984). Muto and Goodman (1972) also demonstrated the loss of correlation between serum vitamin A and total RBP levels as vitamin A became negligible, presumably since apo-RBP still circulated albeit in a denatured form, supporting the findings in the present study. Alternatively, Muto and co-workers (1972) suggested that there was a time lag of 3 days between a decline in serum vitamin A levels, and a corresponding decline in serum total RBP levels. Therefore, if serum measurements were determined within 3 days of a

-242-

decline in serum vitamin A level, serum total RBP level would remain higher.

The relationship between serum total RBP and Vitamin A within an individual may be more complex than previously thought. Although a good correlation between serum levels was well reported (Smith et al., 1970, Smith & Goodman, 1971, Vahlquist et al., 1978), this was not consistent at low serum vitamin A levels (Muto & Goodman, 1972). The correlation might also be lost during transient falls in serum vitamin A level in healthy individuals. The effects of ingestion of vitamin A on RBP synthesis in patients with liver disease requires further investigation, however the findings in the present study indicate that vitamin A may be able to utilise less specific serum binding sites, or circulate in increased quantities in the form of esters in the absence of RBP (James et al., 1984).

x)   Summary.

Although the serum total RBP levels found in chronic liver disease fell just within the previously reported normal range, they were significantly lower that those found in the liver transplant recipients. Assuming that the serum total RBP levels were higher than expected in the present study, the serum levels obtained from patients with chronic liver disease were likely to represent serum total RBP levels much lower than would be observed in a healthy

individual. Furthermore, Shenai and co-workers (1981) suggested that a serum total RBP level of 30mg/l was adequate, and serum total RBP levels lower than this were found in patients with EHBA following an unsuccessful Kasai, cirrhosis, primary biliary cirrhosis and abeta-lipoproteinaemia in the present study. Moreover, even if the serum total RBP level in patients with chronic liver disease or cystic fibrosis did fall within the normal range, a proportion might represent denatured apo-RBP, unable to bind with vitamin A (Large et al., 1980).

In the absence of RBP, vitamin A may still reach the serum in the form of vitamin A esters, and this may result in toxicity due to unregulated transport of the vitamin A (Vahlquist et al., 1982).

CHAPTER 10: THE IMPRESSION CYTOLOGY TECHNIQUE.

i)   The impression cytology technique.

The results obtained from the impression cytology technique were very disappointing. The cellulose ester impressions shown in Figure 10.2 were all obtained from a healthy adult control with a serum vitamin A level of $2.87\pm0.16\mu mol/l$ (n=3), who had no evidence of vitamin A deficiency.   The strip of cellulose ester had been applied to the conjunctiva, as shown in Figure 10.1 to demonstrate the regional variation of goblet cells (Thoft et al., 1984).
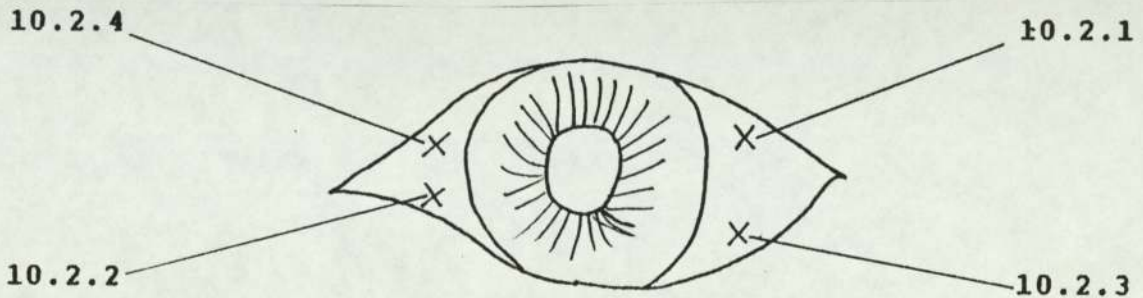
Figure 10.1. shows the position of the filter strip used to gain the impressions of conjunctival epithelial cells shown in Figure 10.2.

The impression obtained from the temporal bulbar conjunctiva (Figure 10.2.1) showed distinct goblet cells, along with small densely packed epithelial cells. Likewise, small densely packed epithelial cells along with a large number of goblet cells were observed in the impression from the inferior nasal quadrant (Figure 10.2.2). Impressions from the superior nasal and outer lower region of the conjunctiva showed larger epithelial cells and no goblet cells (Figures 10.2.3 & 10.2.4).

-246-
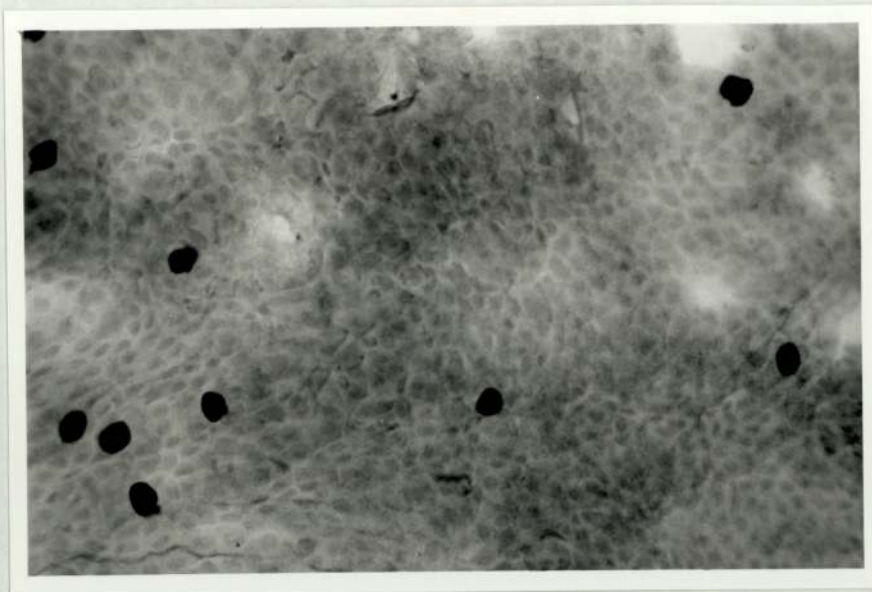
Figure 10.2.1. shows a specimen of epithelial cells taken
from the temporal bulbar conjunctiva of a healthy adult
control (Serum vitamin A level = 2.87±0.16μmol/l) (n=3), by
the impression cytology technique. Densely packed small
epithelial cells with large nuclei are present, with
distinct goblet cell secretions which were strongly PAS-
positive. (PAS-hematoxylin, original magnification x 100).
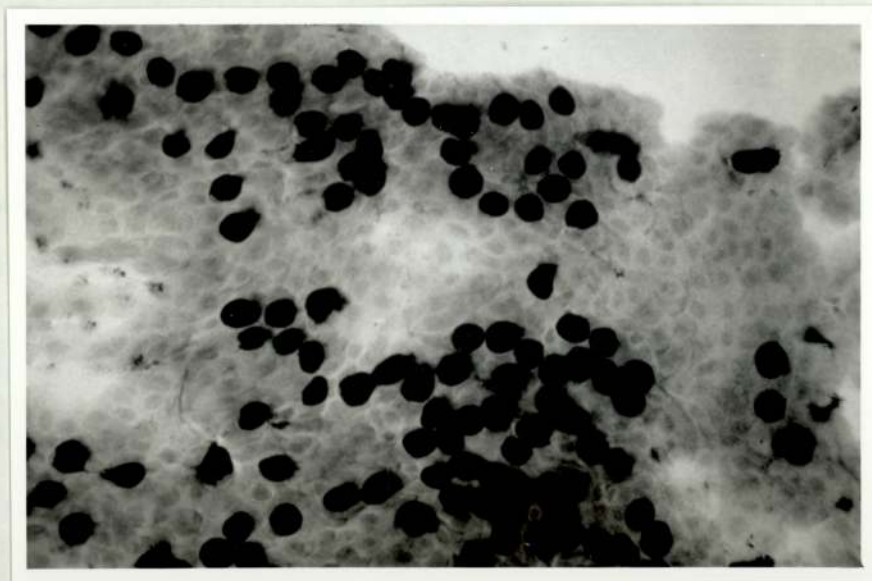
Figure 10.2.2. shows a specimen of epithelial cells taken
from the inferior nasal quadrant of the conjunctiva in a
healthy adult volunteer (as above), by the impression
cytology technique. A large number of strongly PAS positive
goblet cell secretions were present along with small densly
packed epithelial cells containing large nuclei. (PAS-
hematoxylin, original magnification x 100).
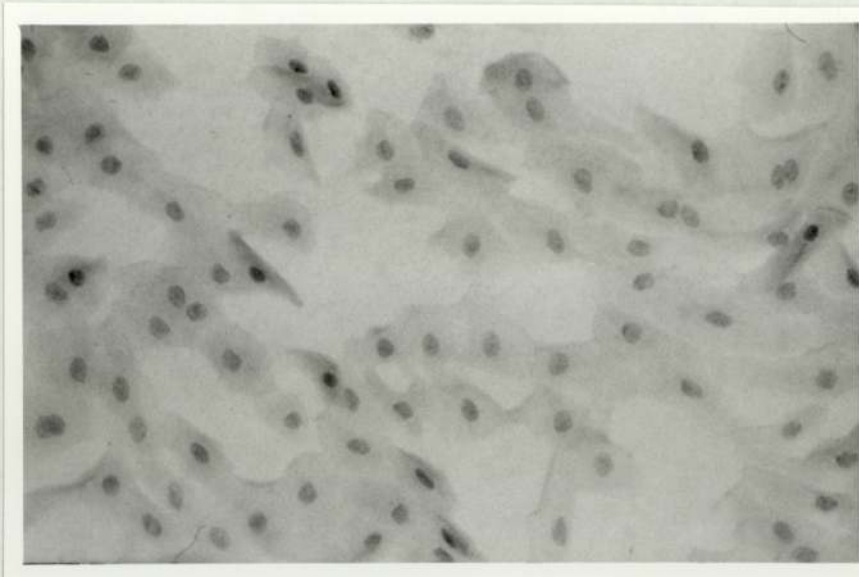
Figure 10.2.3. shows a specimen or epithelial cells taken
from the superior nasal conjunctiva of a healthy adult
control (mean serum vitamin A level = 2.87±0.16umol/1)
(n=3). Epithelial cells appeared enlarged and mishapen with
smaller nuclei than seen in **Figures 10.2.1 or 10.2.2**. Goblet
cell secretions are completely absent. (PAS=hematoxylin,
original magnification x 100).
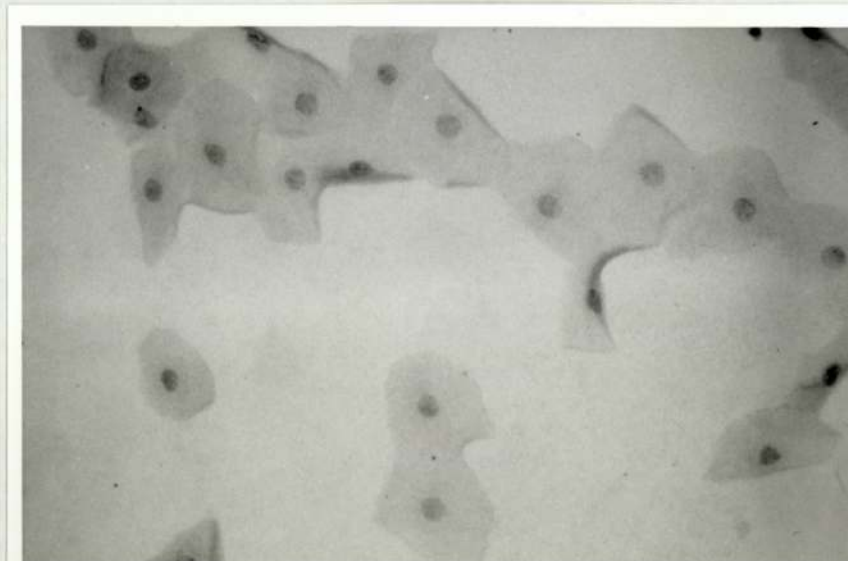
Figure 10.2.4 shows a specimen of epithelial cells taken
from the lower region of the bulbar conjunctiva of a healthy
adult control (as above) using the impression cytology
technique. A small number of enlarged epithelial cells with
small nuclei are present, with no evidence of goblet cell
secretions. (PAS-hematoxylin, original magnification x 100).

If the system for grading conjunctival morphology was applied to the impressions obtained from the healthy adult, the impressions shown in Figures 10.2.1 & 10.2.2 would be classified as grade 0, or normal, however the impression in Figure 10.1.3 & 10.1.4 would more closely fit the grade 2 or 3 classification, indicating vitamin A deficiency (Nelson et al., 1983).

Since interpretation of the impressions remained difficult, and the technique was very time consuming in an out-patient clinic setting, the technique was not pursued (see chapter 11.i.7.)

ii) Discussion

Clearly, incorrect positioning of the cellulose ester strip could considerably influence the results obtained from the impression cytology technique (see above). Although the filter strips were positioned with care, they might slip, for example if the patient blinked or rolled the eye. Such a response was frequent, since it was a natural reflex when a foreign body approached the eye. Moreover, since the application and removal of the filter strip was rapid, it was difficult to determine the exact position of the filter strip. Application of insufficient pressure to the strip on the conjunctiva might also give the impression of subclinical vitamin A deficiency, as a result of insufficient sampling.

Although goblet cells were more frequent in impressions from the inferior nasal quadrant (Figure 10.2.2) this area was difficult to sample, since positioning of the filter strip required the investigators fingers to be directly in the patient's line of vision. This was very disconcerting and patient co-operation was reduced. The temporal bulbar conjunctiva could be approached from the side of the patient and was much more acceptable. Furthermore, although the goblet cells were less frequent, they were clearly visible.

Impressions such as those seen in Figure 10.2.3 or 10.2.4 were frequently obtained from healthy adult controls, since precise positioning of the cellulose ester strip was not always possible. With experience problems associated with the positioning of the strip might be overcome, however the time involved in obtaining the impression was likely to remain prolonged, particularly when working with young children who were unable to co-operate fully. Although the technique might have some potential, an extensive investigation would be required to establish a reliable technique, and this was beyond the scope of the present study. The technique was therefore not routinely applied to patients included in the present study.

PART IV : DISCUSSION.

CHAPTER 11 : CONCLUDING DISCUSSION.

i)   Biochemical indicators of vitamin A deficiency.

Vitamin A status represents the product of processes involving intake, storage, mobilisation, utilisation, and excretion of vitamin A (Underwood, 1984). In a healthy individual, factors affecting vitamin A status are constantly changing. For example, there is seasonal variation in the availability of vitamin A-active foods (Ziegler et al., 1987), in the incidence of diarrhoeal, respiratory (De Sole et al., 1987, Milton et al., 1987) and infectious diseases (Aron et al., 1946, Sivakumar & Reddy, 1972), and in growth rates (West et al., 1988). The balance of these factors is complex, resulting in considerable difficulties in assessing vitamin A status.

The use of various biochemical indicators of vitamin A status has aroused much debate (Underwood, 1984, Olson, 1987, 1982, Chopra & Kevany, 1970). No single, practical and sensitive measure of vitamin A status has been established. The indicators of vitamin A status have been discussed below in terms of their relative merits, disadvantages, and their practical applications, with particular reference to the present study:

1.   Dietary assessment.

2.   Serum vitamin A levels.

3.   The relative dose response test.

4.   Serum retinol-binding protein levels.

-253-

5.   Hepatic vitamin A concentration.

6.   Hepatic retinol-binding protein concentration.

7.   Impression cytology technique.

8.   Physiological indicators eg. dark adaptation.

1.   Dietary Assessment

Interpretation of dietary assessment, unless assessing a weighed dietary intake, has often been difficult (Underwood, 1984). A 7-day assessment was thought to be more comprehensive than a shorter period such as 3 days, particularly for fat intake which varies considerably from day to day. However, patient compliance with the assessment might be poor. Moreover, in malabsorptive states a dietary assessment could not distinguish between vitamin A-active foods ingested, and the actual proportion absorbed.

In the present study, the mean dietary intake of vitamin A-active foods including any vitamin A supplements was found to be four times the recommended daily intake in patients with chronic liver disease. Only 1 of the patients demonstrated an intake of vitamin A less than 100% of the recommended daily intake. Despite this, 8 of the 12 patients had serum vitamin A levels < 1.0μmol/l, 3 being < 0.70μmol/l).

The data obtained in the present study emphasised that assessment of vitamin A intake did not reflect that which was absorbed and transported in malabsorptive states.

-254-

Moreover, dietary intake of vitamin A active foods did not appear to be poor in chronic liver disease, contrary to previous reports (Mezey, 1987). These findings indicated that vitamin A was either being malabsorbed or not being transported, since serum vitamin A levels remained low despite adequate dietary intake of vitamin A.

2.   Serum vitamin A levels.

The limitations in the measurement of serum vitamin A levels, with respect to specificity and sensitivity have been widely accepted (Underwood, 1984, Olson, 1987). In addition to vitamin A status, serum vitamin A levels were influenced by the general nutritional status of the individual (Smith et al., 1973a), were reduced in infection (Mendez et al., 1959, Sivakumar & Reddy, 1972) or stress (Moritai & Nakano, 1982), and were under homeostatic control (Amedee-Manesme et al., 1985). Furthermore, serum vitamin A levels were maintained at the expense of hepatic vitamin A stores, therefore a deficiency might not be recognised until hepatic stores were severely depleted. In view of these limitations, serum vitamin A levels might not be a reliable indicator of vitamin A status (Rao et al., 1987), except when the concentration was very low ($< 0.35\mu$mol/l) (Amedee-Manesme et al., 1985).

however there was little data to support the actual incidence of overt clinical symptoms at these serum levels (Underwood, 1978). The range of serum levels between 0.70-0.99μmol/l have been termed "marginal", that is to say that overt clinical symptoms were unlikely (Underwood, 1978). The actual significance of such serum vitamin A levels was not known, however subclinical vitamin A deficiency might be present (WHO, 1982, Chandra, 1988).

b. Serum vitamin A levels in healthy individuals.

The normal range for serum vitamin A and carotenoid levels might be expected to be dependant on the analytical method employed for the determination of serum levels. Although the HPLC technique has been reported to be the most sensitive analytical method (Frolick & Olson, 1984), a good correlation was found when specimens were tested simultaneously in the present study, using colorimetry (Neald & Pearson, 1963) and HPLC (Thurnham et al., 1988); the intra-assay variation was calculated as 2% for vitamin A (5% and 4% respectively for carotenoids). The HPLC method had the advantages of requiring a smaller specimen volume (0.3ml), distinguishing between the different carotenoids, and being able to simultaneously determine vitamin E. However the technique was considerably more expensive than the colorimetric assay employed in the present study.

-257-

The literature contains a wealth of information on serum vitamin A levels in healthy individuals, and the effects of age on these serum levels. The data has been summarised in Table 11.1, with particular reference to the colorimetric assay.

Age	Serum Vit A Mean (μmol/l)	Serum Vit A Range (μmol/l)	Reference
Premature	>0.35		Woodruff et al., 1986, Kahan, 1969.
Newborn	0.77	0.24-1.30	Lentner, 1984, High, 1969 O'Neil et al., 1970,
1-6 months	1.00		Kahan, 1969, High, 1969, O'Neil et al., 1970.
6 months -10 years	1.00		Kahan, 1969, High, 1969, O'Neil et al., 1970, Owen et al., 1971.
10-13 years	1.54	0.70-2.30	Lentner, 1984, O'Neil et al., 1970.
Adults[*] male	3.40	2.10-4.70	Eastham, 1985.
female	2.90	1.50-4.10	Eastham, 1985.
Adults	1.13	0.72-1.64	Lentner, 1984.
	1.75	0.65-2.85	Lentner, 1984, O'Neil et al., 1970, Carney & Russell, 1980, Garry et al., 1987.

Table 11.1 Serum vitamin A levels in healthy individuals. There were numerous adult ranges quoted in the literature, those listed were determined using the colourimetric assay, with the largest number of patients. The difference between males and females was not universal, and was not thought to occur to any significant extent in children (High, 1969, O'Neil et al., 1970.

The aim of vitamin A supplementation in patients with malabsorptive states, was to provide sufficient vitamin A to maintain all of the physiological actions of vitamin A (see chapter 2.iv.). As previously discussed, tissue requirements for vitamin A may vary (Robrigues & Irwin, 1972), however in terms of serum vitamin A levels it appeared sensible to aim for serum levels equivalent to those found in a healthy individual, of similar age. Unfortunately no distinction between what was necessary, and what was observed in a healthy individual has ever been made. However, Eastham (1985) stated that there was no risk of clinical symptoms of vitamin A deficiency at serum vitamin A levels > 1.40μmol/l. Moreover, Kaufman and co-workers (1987) recommended attainment of serum vitamin A levels between 1.40-1.70μmol/l in chronic liver disease.

Since many of the patients in the present study were young children, and would therefore be expected to have lower serum vitamin A levels than adults (See Table 11.1), serum vitamin A levels > 1.00μmol/l were considered adequate. Although serum levels of 0.70-1.00μmol/l might be observed in healthy individuals, for example during times of infection (WHO, 1982), the aim in the present study was to maintain the serum vitamin A levels > 1.00μmol/l, since previous investigators suggested that subclincal vitamin A deficiency might be associated with persistent serum levels of 0.70-1.00μmol/l (Underwood, 1984, Chandra, 1988).

c.   Data obtained in the present study.

The problems encountered in the present study, with age-matched controls have been described in chapter 1. The serum vitamin A levels obtained from the healthy adult controls and liver transplant recipients correlated well with the normal ranges quoted in Table 11.1. Although only 8 liver transplant recipients were included in the present study, the mean serum vitamin A level represented the mean of 30 separate specimens. This compensated for any fluctuations in the serum levels, associated with transient infection (WHO, 1982), or seasonal variation in dietary vitamin A intake (Ziegler et al, 1987). The serum vitamin A levels were found to be consistently between 1.00-1.40μmol/l, therefore vitamin A supplementation was considered adequate in patients with malabsorptive states, if serum vitamin A levels were consistently between 1.00-1.40μmol/l.

If the number of patients with serum vitamin A levels < 0.70μmol/l and < 0.35μmol/l in the present study was considered, biochemical vitamin A deficiency did not appear to be a problem in patients with cystic fibrosis, receiving a standard daily dose of vitamin A (see chapter 1.i.). However, 30% of the patients with chronic liver disease, who were receiving a daily vitamin A supplement of 2,500iu were found to have a mean serum level < 0.70μmol/l, 9% being < 0.35μmol/l. If the patients with EHBA, following an unsuccessful Kasai operation were regarded separately, the

-260-

risk of vitamin A deficiency was considerable, 73% of patients having serum vitamin A levels < 0.70µmol/l and 27% having serum levels < 0.35µmol/l.

The high proportion of patients with chronic liver disease found to have serum vitamin A levels < 0.70µmol/l supported the view that supplementation with a fixed daily dose of 2,500iu vitamin A was inadequate. Moreover the 27% of those patients with EHBA, in whom the Kasai operation had been unsuccessful with serum vitamin A levels < 0.35µmol/l would be expected to develop clinical signs of vitamin A deficiency, including xerophthalmia (Amedee-Manesme et al., 1985). Patients with chronic liver disease therefore appeared to be at risk of developing vitamin A deficiency, and merited consideration of increased vitamin A supplementation.

d. Consideration of serum vitamin A levels in malabsorptive states.

Since the limitations associated with measurement of serum vitamin A levels had to be accepted (see above), careful interpretation was required if any valuable information was to be gained from measurement of serum vitamin A levels. The following factors were considered:

a. Malabsorption was likely in the patients included in the present study, and although a dietary assessment might indicate adequate intake of

-261-

vitamin A, the actual amount absorbed was likely to be reduced considerably.

b. Malabsorption was a long term problem, and unlike an infection or a seasonal decrease in vitamin A intake in a healthy child, would not be self-limiting unless the disease state improved significantly. Moreover, the duration of a transient drop in serum vitamin A level, resulting from an infection or seasonal decrease in vitamin A intake would not be expected to create a problem in a healthy individual, unless infection was recurrent (Sivakumer & Reddy, 1972).

c. Although variation in serum vitamin A levels was observed in the patients with chronic liver disease included in the present study, serum levels were relatively consistent, confirming that the lower serum vitamin A levels found were not transient. These persistently low serum vitamin A levels might be associated with the development of vitamin A deficiency.

3. The relative dose response test.

Several investigators have suggested that measurement of a relative response to a dose of vitamin A, was of more value in determining vitamin A status than a single serum level (Amedee-Manesme et al., 1987, Loerch et al., 1979).

Initial studies in rats demonstrated that the pattern of response to a small dose of vitamin A over 5 hours, could differentiate rats with hepatic vitamin A stores of 10μg/g, from those with hepatic stores of 20-30μg/g or > 30μg/g (see below) (Loerch et al., 1979). Both hepatic and serum vitamin A levels had to be reduced for the test to be reproducible. The basis for this approach consisted of several fators:

a.   In vitamin A deficiency, apo-RBP accumulates in the liver; upon the administration of a small dose of vitamin A the RBP was rapidly mobilised, as holo-RBP (Muto et al., 1972).

b.   Furthermore, in vitamin A deficency the preferred route for newly ingested vitamin A appears to be immediate mobilisation, rather than hepatic storage (Loerch et al., 1979, Yeung & Veen-Baigent, 1974).

c.   If hepatic vitamin A stores are adequate, ingestion of a small dose of vitamin A causes little response in serum vitamin A level (Loerch et al., 1979).

The relative dose response (RDR) was defined as the percentage difference between serum vitamin A levels at 0hr and 5hr, following ingestion of a small dose of vitamin A. The relative dose response test has been applied to patients

-263-

with chronic liver disease (Amedee-Manesme et al., 1987b), patients with normal, and low serum albumin levels (Russell et al., 1982), and patients postulated to have marginal vitamin A status (Flores et al., 1984). A relative dose response of > 20% was taken as indicative of hepatic vitamin A stores < 20µg/g (Flores et al., 1984, Amedee-Manesme et al., 1987b).

Amedee-Manesme and co-workers (1984a) calculated the relative dose response in patients with cholestasis and hepatic vitamin A stores > 30µg/l as < 15%, whereas patients with hepatic stores of 14µg/l had a relative dose response of 28%. Hepatic stores of < 10µg/g were associated with relative dose response > 67%. Treatment with 200,000iu vitamin A resulted in relative dose response < 20% in 14 of 23 patients with cholestasis (Flores et al., 1984).

If retinol-binding protein synthesis/release was impaired, there might be no response to a dose of vitamin A, since vitamin A could not be transported. This might result in a relative dose response < 15% (Russell et al., 1978). Since the hepatic stores of vitamin A might appear adequate in such patients, as vitamin A accumulated in the liver (Underwood & Denning, 1972), correlation with hepatic stores would remain (Amedee-Manesme et al.,1984a). However, this vitamin A would be inactive, since it could not reach the site of action (Russell et al., 1978).

-264-

The relative dose response test was determined in the present study, in 4 subjects with chronic liver disease and 5 adult controls, who underwent absorption tests. Although the dose administered (5,000iu or 10,000iu) and the vitamin A preparation (water-miscible liquid) differed from previous studies (Flores et al., 1984, Amedee-Manesme et al., 1984a), a similar response was noted. This indicated that the adult controls had adequate hepatic vitamin A stores (RDR < 20%), whilst the subjects with chronic liver disease had reduced hepatic stores (RDR > 30%).

The relative dose response was high (56%) in one patient who received 10,000iu of vitamin A, despite a low serum RBP level (9mg/l), indicating that the relative dose response test might be independent of RBP status. Although confirmation would be required, this result contradicted the findings of Russell and co-workers (1978). Serum total RBP levels were not available for the remaining patients, however 2 suffered from EHBA and the Kasai operation had been unsuccessful, therefore low serum total RBP levels would be expected (see chapter 9.iv.). In the final patient, the serum vitamin A level at the time of relative dose response test determination was 0.37μmol/l, indicating that hepatic vitamin A stores were depleted (Underwood, 1984).

In conclusion, the relative dose response test would probably be more suitable than an absorption test to investigate absorption of vitamin A, since it required only

-265-

two blood specimens. Furthermore, Fulton and co-workers (1982) demonstrated a correlation between the relative dose response and dark adaptation. If this finding was substantiated, the relative dose response could be used to estimate the risk of impaired dark adaptation in young children, who could not undergo ERG or dark adaptation examination. This correlation was confirmed by Mobarhan and co-workers (1981), however although dark adaptation responded to treatment with oral vitamin A, the relative dose response did not, and the correlation was lost. Conversely, Russell and co-workers (1978) did not find a correlation between dark adaptation and the relative dose response where RBP status was abnormal.

As a screening technique, the usefulness of the relative dose response was limited by the need to wait 5 hours between specimen collection. Moreover, the effect of RBP status on the test required confirmation.

4. Serum total retinol-binding protein levels.

In serum, the molar ratio of vitamin A:RBP has been found to be approximately 1:1 (Peterson, 1971), with little apo-RBP (not bound to vitamin A) being present. Serum total RBP levels could therefore be used as an indicator of vitamin A status, since it was required for vitamin A transport to the sites of action (Kanai et al., 1968). Measurement of serum total RBP also required a much smaller specimen volume (50-

100μl) of serum, and RBP was much more stable than vitamin A during storage (Dawson, 1986).

However, serum total RBP levels were sensitive to short term dietary protein and calorie malnutrition, lower serum levels occurring even in mild protein energy malnutrition (Ingenbleek et al., 1975). Moreover, the correlation between vitamin A and total RBP was lost at low serum vitamin A levels (Large et al., 1980, Amedee-Manesme et al., 1985). This might result in overestimation of vitamin A status, unless distinction between serum holo- and apo- RBP was made (Glover et al., 1974). Therefore, whilst serum total RBP levels provided useful information in conjunction with serum vitamin A levels, the technique was of little value as a single screening technique. Furthermore, the serum RBP levels in the present study appeared to be higher than had been previously reported (see chapter 9.ix.2.). This may have resulted from prolonged storage of the standard serum. Alternatively, it may indicate that assay variation occurs, and therefore comparison with the work of other investigators would be difficult.

5.   Hepatic vitamin A concentration.

Hepatic vitamin A concentration has not generally been used as a screening technique for vitamin A status (Olson, 1978), although analysis of liver tissue has been completed at biopsy (Amedee-Manesme et al., 1985) and

autopsy (Congden et al., 1981, Underwood & Denning, 1972).
Several investigators suggested that this was the most
accurate single determinant of vitamin A status, however it
was an invasive technique (Olson, 1978, Underwood & Denning,
1972, Congden et al., 1981). Therefore, although the
technique was found to be a relatively simple and safe
procedure, it was not ethically justifiable as a screening
technique (Olson, 1978). Vitamin A status has been defined
in terms of hepatic vitamin A concentrations as shown in
Table 11.2 (Olson, 1978, Goodman, 1984a).

Vit A Status	Hepatic Vit A Concentration[*] (µg/g)
Adequate	> 20
Marginal	10-20
Poor	5-10
Critical	< 5

[*] There was considerable variation in the distribution of
vitamin A in the liver (MacClaren et al., 1979), therefore
specimens were always taken from the centre of the right
lobe, where the concentration was highest.

Table 11.2. Hepatic vitamin A concentration.
-268-

Hepatic vitamin A concentration appeared to be related to age (Olson et al., 1984, Flores & De Araujo, 1984, Amedee-Manesme et al., 1984b). The most extensive study demonstrated a median hepatic vitamin A level of 11µg/g at birth, increasing rapidly to the age of four years, after which it remained relatively constant (Olson et al., 1984). Since 2/3 of healthy infants had hepatic vitamin A concentrations < 20µg/g, the criteria for vitamin A status shown in Table 11.2 seemed inappropriate in children under 6 months old.

Many of the patients in the present study were less than 6 months old, therefore determination of hepatic vitamin A concentration would yield little useful information. Moreover, accumulation of vitamin A within the liver has been demonstrated in cystic fibrosis, as a result of defective RBP mediated transport (Underwood & Denning, 1972). If hepatic vitamin A concentration was used as a single screening technique, such an effect would indicate good vitamin A status, however vitamin A deficiency could still occur, since vitamin A could not reach the sites of action. Furthermore, liver biopsy was not routine in all patients included in the present study, and as discussed above, it would not be ethically justifiable to obtain a biopsy to screen vitamin A status, unless clinically indicated.

6.   Hepatic retinol-binding protein concentration.

Although hepatic RBP concentration has been measured,
applications to clinical studies were poorly defined. In
vitamin A deficiency, accumulation of RBP was observed in
patients with cystic fibrosis, and this might result in
overestimation of vitamin A status (Underwood & Denning,
1972). Detailed studies have been completed in rats,
demonstrating a very close correlation between serum and
hepatic RBP in conditions of deficiency and repletion (Smith
et al., 1973a, Muto et al., 1972).

Utilisation of radioisotopes might allow labelling of
pre-RBP, the precursor for hepatic RBP synthesis. Such a
technique might provide evidence indicating whether the low
serum RBP levels resulted from reduced hepatic synthesis or
release of RBP, or were a product of reduced vitamin A
availability (caused by malabsorption).

Further investigation of the technique is required, but
determination of hepatic RBP concentration might
significantly contribute to the assessment of vitamin A
status. However, since it is an invasive test the
applications as a screening technique would be limited.
Furthermore, since hepatic accumulation of RBP has been
noted in vitamin A deficiency, reliance on the technique for
screening vitamin A deficiency might result in
overestimation of vitamin A status.

-270-

7.   Impression cytology.

In the impression cytology technique, an absorbant
filter was applied to the temporal bulbar conjunctiva, to
obtain a sample of epithelial and goblet cells (Amedee-
Manesme et al., 1988). Each specimen was stained and
examined for the presence or absence of goblet cells, and
enlarged, flattened epithelial cells. The technique
determined the effect of vitamin A status at a site of
action, allowing consideration of the ability of the patient
to absorb, transport and utilise vitamin A. Amedee-Manesme
and co-workers (1988) suggested that the technique could
detect early, reversible subclinical vitamin A deficiency.
As a screening procedure, the technique demonstrated several
advantages; it was non-invasive, required no specialist
equipment and was inexpensive. In addition, the early
changes in conjunctival morphology were found to be
reversible, if vitamin A deficiency was corrected, therefore
treatment could be monitored (Hatchell & Sommer, 1984,
Amedee-Manesme et al., 1988).

The results obtained in the present study were however,
very disappointing. Firstly, a regional variation of goblet
cells on the conjunctiva has been established (Thoft et
al., 1984), therefore if the filter strip was incorrectly
positioned, apparent reduction in goblet cell number might
result from experimental error, rather than subclinical
vitamin A deficiency. A high proportion of the specimens

-271-

obtained from healthy adult controls in the present study appeared to demonstrate abnormal conjunctival morphology, indicative of subclinical vitamin A deficiency, presumably as a result of operator inexperience. Secondly, the technique required subject co-operation, and was therefore difficult in children.

The technique was found to be very time consuming, attainment of the specimen taking 15-30mins, followed by a further 30 minutes to stain and mount the specimen. As a research tool this might not present a problem, however in a busy out-patient clinic where the average time per patient was approximately 15 minutes, it was clearly inpractical. These problems might be overcome, to some extent with experience therefore the technique cannot be dismissed.

A further consideration was that impression cytology was specific to the ocular effects of subclinical vitamin A deficiency, and did not allow consideration of the adverse effect on growth, or resistance to infection. Since tissue requirements for vitamin A differ, these effects could not be assumed to be related to changes in conjunctival morphology (Robrigues & Irwin, 1972). In conclusion, the impression cytology technique was perhaps more specific than serum vitamin A levels for the evaluation of vitamin A status, however the specificity was such that only a single effect of vitamin A deficiency was considered. Therefore, although the information was useful, complete evaluation of

-272-

vitamin A status was still not possible.

8.   Dark adaptation and electroenterographic examination.

Physiological tests for vitamin A deficiency such as dark adaptation or electroretinographic examination (ERG), were only specific to vitamin A deficiency in young children (Genest et al., 1967). Moreover, patient co-operation was required, therefore accuracy of the test might be limited in young children, although Alvarez and co-workers (1981) suggested that the ERG could be used in children as young as 6 months old. However, this still excluded a large number of patients in the present study, particularly those with EHBA who had demonstrated the lowest serum vitamin A levels. Such patients were therefore at the greatest risk of developing subclinical vitamin A deficiency.

As a screening technique, dark adaptation or ERG lack specificity, except in young children. Moreover the application in very young children was poor, and complex apparatus was required. In addition, reversal of abnormal dark adaptation might not be possible, further limiting usefulness as a screening technique.

9.   Conclusions.

All of the above biochemical and physiological indicators of vitamin A deficiency were found to be limited with respect to specificity and/or sensitivity, or practical

application. Serum levels of vitamin A, along with additional information from determination of serum RBP levels remained the most practical means of assessing vitamin A status, provided the limitations were acknowledged. Particulary if techniques were developed to distinguish between serum vitamin A and vitamin A esters (James et al., 1984) and holo- and apo-RBP (Glover et al., 1974). This would ensure that use of high dose vitamin A supplements was not likely to be associated with toxicity, as a result of increased serum concentrations of the vitamin A esters, and might also allow more detailed investigation of any possible defect in RBP synthesis or metabolism. Further investigation of the impression cytology technique and measurement of hepatic RBP concentration may well provide invaluable information on vitamin A status.

ii) Interpretation of serum vitamin A levels.

Interpretation of serum vitamin A levels requires realisation that only persistently low serum vitamin A levels are associated with vitamin A deficiency. A transient drop in serum vitamin A levels in a healthy individual would not be associated with signs of vitamin A deficiency. Since the prevalence of clinical signs of vitamin A deficiency was not investigated in the present study, such persistently low serum vitamin A levels had to be taken as indicative of risk, rather than absolute proof of vitamin A

-274-

deficiency. As the serum levels fell, and remained low so the risk of developing vitamin A deficiency increased.

Since serum vitamin A levels were influenced by many factors (see section i.), an absolute relationship between serum levels and vitamin A status could not be found. Furthermore, although the association of persistently low serum vitamin A levels and vitamin A deficiency was well established, the relationship between the actual duration of the low serum vitamin A levels and the development of vitamin A deficiency remained highly individual. This might be related to the amount of vitamin A stored in the liver prior to illness, the actual pathophysiology underlying the vitamin A deficiency (ie malabsorption and/or defective RBP synthesis/release), and possibly the varying ability of tissues to utilise vitamin A in a disease state (Mezey, 1978). Moreover, the onset of vitamin A deficiency might be hastened by other factors such as recurrent infection which increase tissue requirements (Sikakumar & Reddy, 1972).

The data describing the relationship between serum vitamin A levels and vitamin A deficiency has been summarised in Figure 11.1, adapted from Underwood (1984) and Bauernfield (1980). As previously discussed, consideration of the duration of the serum levels would be required to allow full interpretation of serum vitamin A levels; only persistently low serum vitamin A levels would be associated with the symptoms shown in Figure 11.1.

-275-
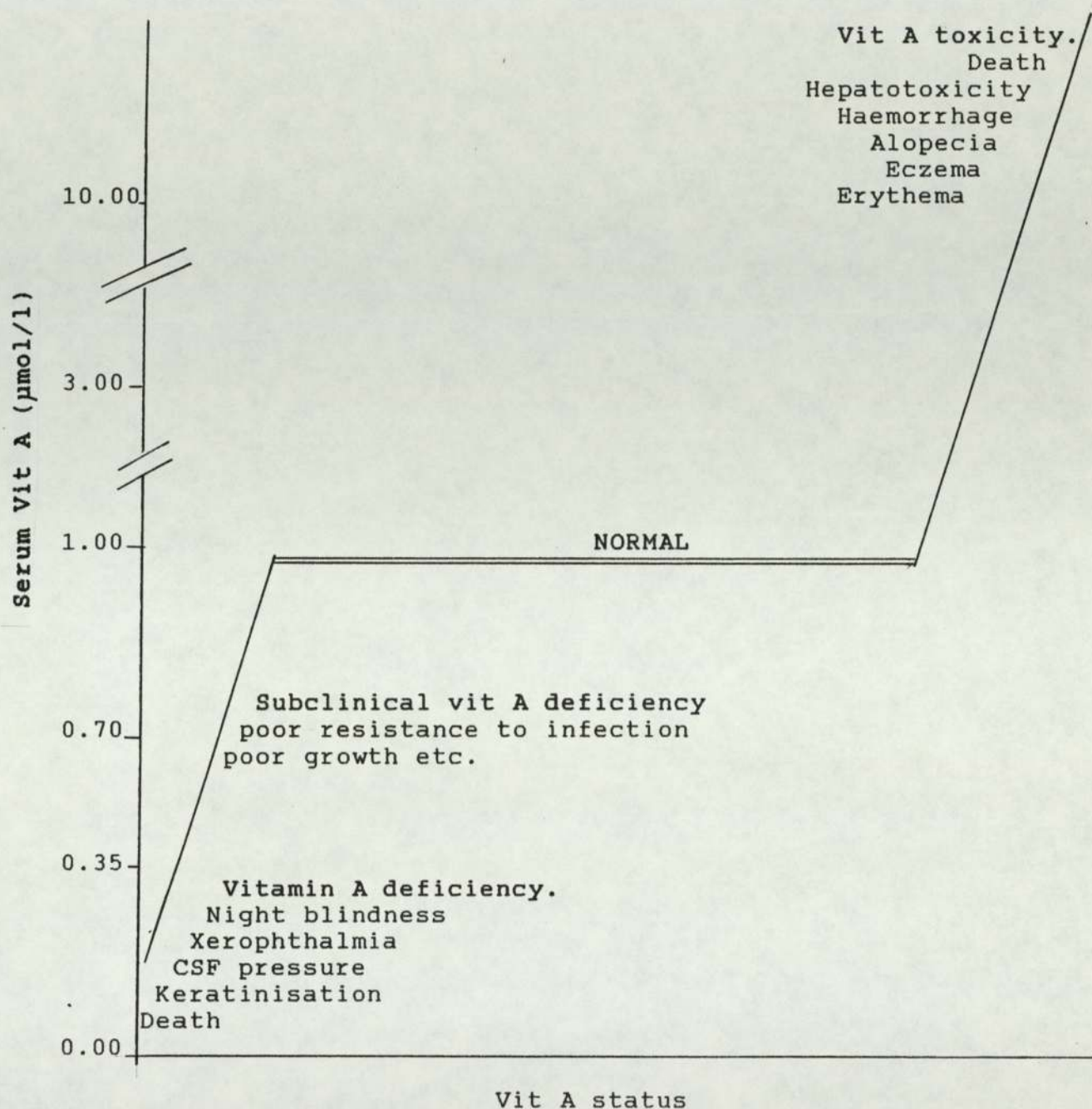
Figure 11.1 represents guidelines for the interpretation of
serum vitamin A levels, adapted from Bauernfield (1980) and
Underwood (1984). The abscissa represents vitamin A status
whilst the ordinate represents serum vitamin A levels
(µmol/l) status. The relationship between serum vitamin A
levels and vitamin A status is also influenced by the
duration of the serum level.

iii) Patients at risk of developing vitamin A deficiency.

Any patient with chronic malabsorption was at risk of developing vitamin A deficiency (see chapter 1.i.). Manifestation as conjunctival or corneal ulceration has not been reported, however reduced dark adaptation (Fulton et al., 1981, Mobarhan et al., 1982) and abnormal conjunctival morphology (Amedee-Manesme et al., 1988), along with reduced growth rates (West et al., 1988) and increased susceptibility to infection might prevail (Milton et al., 1987). The present study indicated that those patients with chronic liver disease, particularly those with EHBA in whom the Kasai operation had been unsuccessful, were at greatest risk of developing vitamin A deficiency, since these were the patients with persistently low serum vitamin A levels.

Furthermore, a significant fall in serum vitamin A levels was found in patients with cystic fibrosis as age increased. This was associated with a change in the dose and preparation of vitamin A from a water-miscible liquid to a capsule which might have resulted in reduced absorption however, infection might have become more frequent with increasing age, and disease state would almost certainly have deteriorated, perhaps causing a further reduction in RBP synthesis/release. Alternatively, if defective RBP synthesis or release was a secondary feature of cystic fibrosis, it might develop later in life causing a fall in serum vitamin A levels (Smith et al., 1972). Serum vitamin A

levels were however, not as low as those demonstrated in
EHBA or chronic liver disease, and since the serum levels
remained > 1.00μmol/1, the clinical implications remained
uncertain. However, the serum vitamin A levels might
represent an increased proportion of unbound vitamin A
esters which are inactive and might ultimately cause
toxicity (James et al., 1984, Vahlquist et al., 1978).

iv) Expression of the risk of vitamin A deficiency.

Although clinical evidence of vitamin A deficiency was
not investigated in the present study, overt signs of
vitamin A deficiency, such as impaired dark adaptation have
been reported in malabsorptive states (Mobarhan et al.,
1981, Fulton et al., 1982, Walt et al., 1984, Russell et
al., 1982, Howard et al., 1982) except EHBA (Andrews et al.,
1981). The lack of reports of overt vitamin A deficiency in
EHBA might have resulted from the inability to detect signs
of vitamin A deficiency, especially in young children, since
the risk of vitamin A deficiency was well documented.
However, application of the impression cytology technique
indicated that the prevalence of subclinical vitamin A
deficiency was probably greater than previously thought
(Amedee-Manesme et al., 1988).

Furthermore, determination of dark adaptation or ERG
examination was not routine, even in the older patients,
therefore signs of vitamin A deficiency might have remained

-278-

unrecognised. In view of these considerations, the frequent falls in serum vitamin A levels justified consideration of vitamin A supplementation (Kaufman et al., 1987, Andrews et al., 1981)

The reason why vitamin A deficiency did not develop fully in patients with malabsorptive states, resulting in xerophthalmia as seen in the third world remained unclear, however vitamin A deficiency in the third world may be exacerbated by recurrent infection, particularly measles (Inua et al., 1983). High dose vitamin A therapy has proved highly successful in the third world, indicating that dietary insufficiency was the prime cause of vitamin A deficiency (Swaminathan et al., 1970, Skirantia & Reddy, 1970). In malabsorptive states, impaired RBP synthesis or release might result in a vitamin A deficiency less responsive to supplementation, since vitamin A could not be transported effectively within the body, regardless of the amount absorbed. However impaired RBP synthesis might also occur in the third world, secondary to protein energy malnutrition (Ingenbleek et al., 1975).

Since 27% of patients with EHBA in the present study were found to have serum vitamin A levels $< 0.35 \mu mol/l$, following an unsuccessful Kasai operation, they were at risk of developing clinical signs of vitamin A deficiency including xerophthalmia. The prognosis for such patients was poor; one died, two received and one urgently awaited a

liver transplant. Since the mean age of the patients was only 15.5±11.6 months (n=4) (range 7-32 months), overt vitamin A deficiency might not develop fully before a patient died or received a liver transplant. Clearly, a number of patients with malabsorptive states were at risk of developing vitamin A deficiency, despite the lack of reports of clinical signs.

v)   Aims of vitamin A supplementation in malabsorptive states.

The aim of vitamin A supplementation in malabsorptive states, should be to eliminate the risk of vitamin A deficiency, without precipitation of toxicity. Eastham (1985) suggested that persistent serum vitamin A levels > 1.4µmol/l were consistent with no risk of developing vitamin A deficiency. However, in younger children serum vitamin A levels > 1.0µmol/l might well be acceptable (see chapter 11.i.2a). Increased vitamin A supplements appeared to be indicated in chronic liver disease, particularly EHBA where the Kasai operation had been unsuccessful, however an upper limit should be placed on the dose, to prevent toxicity resulting from increased serum concentrations of vitamin A esters in the absence of RBP (Vahlquist et al., 1978). Further studies are required to assess such potential toxicity.

vi) Patient compliance.

Patient compliance has not previously been considered in the present thesis, however the influence on the outcome of supplementation was obviously critical. The return of unused medicine was not employed in the present study to establish compliance, since the hospital policy was to provide only the first two weeks supply, further supplies were then obtained from the general practitioner. This rendered determination of the use of medicine virtually impossible. However, parents were asked to recall dosage of all the medicines they had administered at each out-patient appointment, to ensure that drugs were being administered correctly. Patients suspected of non-compliance were excluded from the study. Furthermore, particular attention was paid to explanation to the parents on the reasons for administering vitamin supplements. It was hoped that clear and simple explanations encouraged compliance; parents certainly seemed less likely to dismiss vitamin supplements as unimportant following the explanations.

Compliance was not aided by the complexity of the drug regimens required in some of the patients with chronic liver disease (Table 11.3). Alteration of the dose of vitamin supplements might cause confusion in such a complex regimen. Furthermore, many of the commercially available water-miscible vitamin A preparations were unpalatable, and relatively high volumes were required in order to supply

Time Of Day	Drug	Dose Volume (ml)
Morning	Ranitidine	3
	Gaviscon Liquid	5
	Spironolactone	2
	Ferrous Sulphate	5
	Amoxycillin	5
	Ketovite Liquid	10
Mid-morning	Calcium Sandoz Liquid	5
	Gaviscon Liquid	5
	Ranitidine	3
Afternoon	Ferrous Sulphate	5
	Amoxycillin	5
	Calciferol Liquid	5
	Arovit Drops	5 drops
	Ranitidine	3
Mid-afternoon	Calcium Sandoz Liquid	5
Evening	Ranitidine	3
	Spironolactone	2
	Calcium Sandoz Liquid	5
Night	Ferrous Sulphate	5
	Vitamin K	0.5
	Amoxycillin	5
	Ranitidine	3
	Gaviscon	5

Table 11.3. A typical drug regimen for a patient with chronic liver disease.

increasing doses of vitamin A. In patients undergoing
nasogastric tube feeding this was not a problem, however
this only represented a small proportion of the patients
included in the present study. Arovit drops, containing
5,000iu of vitamin A per drop were considered a more
palatable means of increasing oral vitamin A intake. The
drops could be mixed with a small volume of milk, squash or
feed, prior to the main feed.

Although patient compliance was a potential problem,
practically it did not cause particular concern when parents
were counselled on the importance of supplementation, and
methods for increasing palatability, as described above.

vii) The use of different preparations of vitamin A for
supplementation.

The ideal oral preparation of vitamin A, for
supplementation of patients with malabsorptive states would
contain vitamin A in a form which was readily absorbed.
Furthermore, the concentration would be such that dose
changes were practical, and that higher doses could be
administered, whilst retaining a small volume. Such a
preparation should be palatable, and stable throughout
storage. Although a multivitamin preparation such as
Ketovite liquid was suitable for vitamin A supplementation
in patients with less severe liver disease, and probably
those with cystic fibrosis, palatability was poor. Moreover,

multivitamin supplements were totally inadequate in terms of vitamin A content for those patients with severe liver disease. Even if higher volumes could be utilised to administer a higher dose of vitamin A, compliance would be reduced by the poor palatability.

Although absorption of vitamin A was expected to be reduced in malabsorptive states, there have been several reports of adequate absorption, even with little or no bile flow (Kaufman et al., 1987), and successful treatment with oral vitamin A supplements (Congden et al., 1981, Dutta et al., 1982, Littlewood et al., 1980). In the present study, poor absorption was demonstrated in most of the patients, however a maintenance oral dose of vitamin A resulted in elevation of serum vitamin A levels in patients with neonatal hepatitis, $alpha_1$-antitrypsin deficiency, ultra-short bowel syndrome and abetalipoproteinaemia. Despite apparently similar abilities to absorb vitamin A, the remaining patients with chronic liver disease were unable to attain adequate serum vitamin A levels following regular supplementation. This lack of response probably resulted from the inability to transport the ingested vitamin A, secondary to defective RBP synthesis/release. However, any increase in serum vitamin A levels, even in mild chronic liver disease, might represent an increase in unbound vitamin A esters which might cause toxicity, therefore the increased serum levels observed in patients with chronic liver disease requires further investigation.

Periodic administration of large doses of parenteral vitamin A has been successful in the intervention of vitamin A deficiency in the third world (Underwood, 1984, Skirkantia & Reddy, 1970), however transient symptoms of toxicity such as nausea, vomiting, diarrhoea or headache have been reported (Underwood, 1978). The results of intermittent high dose vitamin A therapy in patients with malabsorptive states appeared to be contradictory. Only two studies have demonstrated the safe and efficacious use of high dose intramuscular vitamin A (100,000iu every 2 months) in EHBA (Amedee-Manesme et al., 1988, Allagille, 1985), and the concern remained that such large parenteral doses might cause hepatotoxicity, particularly in severe liver disease where hepatic storage of vitamin A might be limited (Underwood, 1984, Shepherd et al., 1984). In addition, toxicity might also occur as a result of transient increased serum levels of unbound vitamin A esters (James et al., 1984).

Such an approach could be applied to those patients attending The Children's Hospital, Birmingham, however a decision to employ higher oral doses, rather than parenteral preparations was made since concern over toxicity with high dose parenteral therapy remained; if the oral therapy failed then parenteral therapy could be considered.

viii)   Dosage recommendations for vitamin A.

Specific dosage recommendations for vitamin A remained difficult to make since the requirements of individuals appeared to be highly varied. Continuation of present regimens for infants with cystic fibrosis, patients with gastrointestinal disease (unless particularly severe), or apparently mild liver disease, seemed acceptable. In neonatal hepatitis a dilemma existed, since the outcome of the disease was unclear at the time of diagnosis, and .therefore introduction of vitamin A supplements. Patients could either initially receive high dose vitamin A supplements, reducing the dose if outcome was favourable, or receive a standard daily dose of 2,500iu vitamin A until such time as evidence of cirrhosis became available. The latter approach was probably the most acceptable to avoid any risk of toxicity, however serum vitamin A levels should be monitored regularly so that supplements could be increased rapidly if the serum levels began to fall.

Continued administration of water-miscible preparations of vitamin A might be considered in the older patients with cystic fibrosis. Arovit drops could be utilised since these had a higher concentration of vitamin A than the other commercially available preparations, therefore a smaller volume would be required. The response of serum vitamin A levels, in patients with cystic fibrosis to such a change in supplementation requires investigation.

An absolute dosage requirement for vitamin A in patients with severe liver disease, such as EHBA was not made, however initial doses should probably not be less than 10,000-15,000iu of vitamin A (4-6 times the previous standard supplement). Furthermore, doses should probably be based on the severity and age of onset of the liver diseases, along with the bodyweight of the patient, since the incidence of vitamin A deficiency appears to be related to periods of growth (Underwood, 1984). Once the effect of high dose vitamin A supplements on serum vitamin A and vitamin A ester levels has been established, much higher doses might be employed provided that toxicity was not found to be a problem. Certainly, doses of 25,000iu and 50,000iu did not result in any signs of toxicity in the two young children (under 3 years old) to whom they were administered in the present study, however distinction between serum vitamin A and vitamin A esters was not attempted.

Replacement of a multivitamin preparation with single components is to be considered in patients with severe liver disease. Although this would increase the number of drugs administered to each patient, the doses of the fat-soluble vitamins could be titrated to individual requirements. Hopefully this approach would provide a more comprehensive range of fat-soluble vitamins in adequate doses to such patients. Moreover, more concentrated and therefore more palatable preparations of the vitamins could be utilised.

ix) Implications for other fat-soluble vitamins.

Although all of the fat-soluble vitamins (A, D, E, K) are absorbed via the same route (Passmore & Eastwood, 1986), many factors influence the total body status of these different compounds; RBP was required for vitamin A transport, whereas much of the body's requirement for vitamin D was acquired through synthesis in the stratum granulosum of the skin. In addition, vitamin K could be ingested or be synthesised by intestinal flora, and for reasons which remained unclear the absorption of vitamin E appeared to be less efficient than that of vitamin A.

Since numerous factors influenced body status of the fat-soluble vitamins, it would be difficult to assume status of all of the vitamins based on serum vitamin A levels. However, low serum vitamin A levels indicated that serum levels of the other fat-soluble vitamins might be low (Sokol, 1987). Patients classified as having severe liver disease would be expected to demonstrate prolonged coagulation times, frequently unresponsive to vitamin K therapy and pathological evidence of rickets (associated with vitamin D deficiency). Moreover, low serum vitamin E levels have been associated with chronic liver disease.

In the present study, prolonged coagulation times were found in all patients with EHBA, in whom the Kasai operation had failed, and many following a successful operation. A

further 9 patients had evidence of pathological rickets, 3 suffering from fractures. Serum vitamin E levels remained just within the normal range, despite the very small daily intake of 15mg (as Ketovite tablets). Although the present study did not allow extensive consideration of the other fat-soluble vitamins, the need for investigation was indicated.

FUTURE WORK

x)   Future work.

The present study left many questions unanswered, particularly regarding chronic liver disease in children. Although there have been numerous studies of vitamin A status in adults with chronic liver disease, few dealt with children who appear to be more susceptible to vitamin A deficiency, particularly during periods of growth. Moreover, hepatic storage of vitamin A might not be adequate prior to the earlier onset of disease.

Since the present study indicated that higher doses of vitamin A might be employed in patients with severe liver disease, future studies should probably centre around investigation of possible toxicity resulting from increased concentrations of unbound vitamin A esters in serum. Furthermore, the possible defect in RBP synthesis or release should be investigated to clarify the nature of such a defect, and the relationship of disease states to this defect. Information gained from elucidation of the nature of the defect along with data about the relative influences of cholestasis and cirrhosis on RBP synthesis/release would aid the development of specific dosage guidelines for vitamin A. Calculation of the dose of vitamin A, particularly in chronic liver disease, would require consideration of the age of onset and severity of the liver disease along with the body weight of the patient. Furthermore any dosage regimen would have to be flexible, ensuring dosage could be

changed in response to changes in clinical condition. If high dose oral vitamin a therapy failed, periodic high dose parenteral vitamin A might be considered.

The ELISA assay for serum total RBP also requires further development to investigate the apparently high serum total RBP levels obtained in the present study. Furthermore, assays could be developed in order to distinguish between holo- and apo-RBP, allowing consideration of the possibility of an increased proportion of apo-RBP occurring in the serum of patients with chronic liver disease (Glover et al., 1974). The lack of correlation between serum vitamin A and total RBP levels as vitamin A levels decline could then be investigated more fully. A knowledge of the affinity of apo-RBP and other serum proteins for vitamin A in disease states might answer many of the questions regarding vitamin A transport. The development of an assay for hepatic RBP combined with the utilisation of radioactive isotopes, could be used to confirm whether the poor RBP status resulted from defective synthesis or release of the protein.

Further evaluation of the absorption of vitamin A in malabsorptive states, and the possible utilisation of an alternative pathway for absorption is also required. There is little evidence regarding dose-related absorption of vitamin A, although absorption is thought to be more efficient in vitamin A deficiency (Donoghue et al., 1983). In addition, the effect of vitamin A supplementation on both

serum vitamin A and serum vitamin A ester concentrations requires investigation, due to the potential for toxicity if the proportion of unbound vitamin A esters increases.

In the present study, no estimate of the degree of malabsorption was attempted. Determination of faecal fat or faecal chymotrypsin would allow further investigation of the relative influence of malabsorption on vitamin A status (Kelleher, 1987).

As the use of high dose fat-soluble vitamin supplements has been advocated, the effects of concurrent high doses on absorption requires investigation, particulary since several investigators suggested that vitamin E might modify the absorption of vitamin A (Willett et al., 1983, Yang & Desai, 1977, Kusin et al., 1974). Moreover, Draper (1980) demonstrated that vitamin E enhanced the storage of vitamin A, partially by a anti-oxidant effect at a cellular level, but vitamin E also appeared to exert a sparing effect on vitamin A stores (Hickman et al., 1944). Therefore vitamin E deficiency might result in a reduction in serum vitamin A levels, whilst a moderate dose of vitamin E appeared to enhance the utilisation of vitamin A (Aimes 1969). Robinson and co-workers (1980) also demonstrated a synergistic effect between vitamins A and E in the eye.

Further animal studies are required to establish the full nature of the relationship between vitamins A and E in the body. Until this is elucidated, high doses of vitamin A and E should be administered at different times of the day to avoid any interaction. The administration of vitamin E was however thought to be beneficial, since vitamin E deficiency was likely, particulary in severe liver disease. Furthermore, vitamin E deficiency might exacerbate vitamin A deficiency.

The clinical implications of biochemical vitamin A deficiency, particularly in chronic liver disease should also be considered. Dark adaptation or ERG examination should be completed on patients, particularly those with EHBA (where age permits) and the older patients with cystic fibrosis. Perseverance with the impression cytology technique might yield valuable information on the prevalence of subclinical vitamin A deficiency, and can be applied to those patients less than 6 months old.

CONCLUSIONS

CONCLUSIONS.

The present study demonstrated that a large number of patients, primarily those with early onset severe liver disease such as EHBA in which the Kasai operation had been unsuccessful, or cirrhosis were at risk of developing clinical vitamin A deficiency. Furthermore, the risk of vitamin A deficiency might increase with age in the patients with cystic fibrosis. However the exact clinical implications of this increased risk in cystic fibrosis remained unclear, requiring further investigation of the prevalence of both clinical and subclinical vitamin A deficiency. Initially, dark adaptation or ERG examination should be completed where possible, along with impression cytology to detect subclinical conjunctival changes.

The necessity for increased vitamin A supplements was clearly observed in severe liver disease, however individual requirements varied considerably. Although a clear correlation between serum vitamin A and individual determinants of liver function was not established, serum vitamin A levels did correlate with general clinical status. Patients with moderate to severe liver disease such as EHBA (unsuccessful Kasai operation) or extensive cirrhosis were found to have lower serum levels of vitamin A, despite vitamin A supplementation. Conversely, in mild liver disease such as neonatal hepatitis in which the jaundice was resolving with no evidence of progressive cirrhosis,

-296-

gastrointestinal disease or the younger patients with cystic fibrosis, vitamin A supplementation with a standard regimen seemed acceptable. However, in addition to the severity of disease state, consideration of the time of onset, and therefore existing hepatic vitamin A stores, and the · duration of the disease state was required. Variation in the onset and duration of disease might explain why serum vitamin A levels in moderate liver disease could not be distinguished from those found in patients with severe liver disease.

In addition to malabsorption, vitamin A status was almost certainly affected by the ability of serum RBP to transport vitamin A in chronic liver disease, and this might be the singularly most important aetiological factor since it cannot be treated. The ability of RBP to transport vitamin A may determine the response of an individual to vitamin A supplements, explaining why patients who demonstrated a similar ability to absorb vitamin A did not all respond to regular vitamin A supplementation. Although serum RBP levels remained just within the normal range, severe liver disease resulted in a significant reduction in serum levels, when compared with the liver transplant recipients. As previously discussed, the actual serum RBP levels obtained in the present study might represent a much more serious fall in serum levels than immediately apparent. Moroever, the affinity of the protein for vitamin A might be reduced in malabsorptive states (Large et al., 1981).

In the present study, the lowest serum total RBP levels were associated with severe liver disease such as EHBA where Kasai operation had been unsuccessful, or cirrhosis. These serum levels were less than 30mg/1, and therefore were inadequate for vitamin A transport (Shenai et al., 1981). This confirmed the view that such patients were at greatest risk of developing vitamin A deficiency. In contrast, patients with cystic fibrosis did not have reduced serum total RBP levels, contradicting previous reports (Smith et al., 1972).

Definite dosage recommendations for vitamin A were not made since further information regarding possible toxicity (resulting from increased serum concentrations of unbound vitamin A esters if RBP was unable to transport vitamin A) was required. However, a tentative suggestion that dosage should be linked to the severity of liver disease, has been made. The use of much higher doses, based on body weight with regular monitoring of serum vitamin A levels should be undertaken to establish actual dosage levels. Furthermore the distinction between serum vitamin A and vitamin A esters should be made in order to avoid toxicity; the sucess of such a supplementation regimen may be limited by the ability of RBP to transport vitamin A.

Furthermore, serum zinc levels were found to be borderline in patients with chronic liver disease and cystic fibrosis in the present study. Whilst the

-298-

implications of these serum levels was unclear, zinc supplements might be introduced in those patients who did not respond to vitamin A supplements. However a lack of response was more likely to result from defective RBP synthesis or release.

Severe liver disease and cystic fibrosis was also associated with low serum vitamin E levels. Although no correlation between individual serum levels of vitamin A and vitamin E was found, the serum levels of both vitamins appeared to correlate with the severity of disease state in liver disease. Vitamin E supplements were therefore also indicated, particulary in view of the proposed synergistic action with vitamin A. However, until the effects of high doses of vitamin E on the absorption of vitamin A have been clearly elucidated, the 2 vitamins should be administered at separate times of the day.

As a screening method, measurement of serum vitamin A levels remained the most practical determinant of vitamin A status, although the limitations of the technique were accepted. Continued measurement of the serum levels at regular intervals yielded important information, indicating the outcome of supplementation, however the distinction between serum vitamin A and unbound vitamin A esters should be made to ensure that toxicity does not occur. Valuable information could also be obtained from measurement of serum RBP levels, particularly when the apo- and holo forms can be

separated. Furthermore, in a research setting measurement of hepatic vitamin A and RBP concentration, and the ability to distinguish between serum apo- and holo- RBP would allow elucidation of the aetiology of vitamin A deficiency and the most appropriate means of treatment in malabsorptive states.

The need for considerable future work to fully appreciate vitamin A status in such patients was acknowledged, however the findings of the present study indicated that patients with malabsorptive states, particularly severe chronic liver disease, were at risk of developing vitamin A deficiency. Both malabsorption and defective RBP status were probably involved in the development of low serum vitamin A levels.

REFERENCES

REFERENCES

Abernathy, R.S. (1976). Bulging fontalle as a presenting sign in cystic fibrosis. Vitamin A metabolism and the effect on cerebrospinal fluid pressure. Am. J. Dis. Child. 130, 1360-1362.

Alagille, D. (1985). Management of chronic cholestasis in childhood. Semin. in Liver Dis. 5, 254-262.

Alvarez, F., Landrieu, P., Laget, P., Lemonnier, F., Odievre, M., Alagille, D. (1983). Nervous and ocular disorders in children with cholestasis and vitamin A and E deficiency. Hepatol. 3, 410-414.

Amedee-Manesme, O., Anderson, D., Olson, J.A. (1984a). Relation of the relative dose response to liver concentrations of vitamin A in generally well nourished surgical patients. Am. J. Clin. Nutr. 39, 898-902.

Amedee-Manesme, O., Furr H.C., Alvarez, F., Hadchouel, M., Alagille, D., Olson, J.A. (1985). Biochemical indicators of vitamin A depeletion in children with cholestasis. Hepatol. 5, 1143-1148.

Amedee-Manesme, O., Furr, H.C., Olson, J.A. (1984b). The correlation between liver vitamin A concentration in micro-(needle biopsy) and macro-samples of human liver specimens obtained at autopsy. Am. J. Clin. Nutr. 39, 315-319.

Amedee-Manesme, O., Luzeau, R., Carlier, C., Ellrodt, A. (1987a). Simple impression cytology method for determining vitamin A deficiency. Lancet. 1, 1263.

Amedee-Manesme, O., Luzeau, R., Wittepen, J.R., Hanck, A., Sommer, A. (1988). Impression cytology detects subclinical vitamin A deficiency. Am. J. Clin. Nutr. 47, 875-878.

Amedee-Manesme, O., Mourey, M.S., Hanck, A., Therase, J. (1987b). Vitamin A relative dose response test: validation by intravenous injection in children with liver disease. Am. J. Clin. Nutr. 46, 286-289.

-302-

Amedee-Mansme, O., Mourey, M.S., Therasse, J., Couturier, F., Alvarez, F., Hanck, A., Bernard, O. (1987c). Short and long term vitamin A treatment in children with cholestasis. Am. J. Clin. Nutr. 47, 690-693.

Ames, S.I. (1969). Factors effecting the absorption, transport and storage of vitamin A. Am. J. Clin. Nutr. 22, 934-935.

Andrews, W.S., Pau, C.M.L., Chase, H.P., Foley, L.C., Lilly, J.R. (1981). Fat-soluble vitamin deficiency in biliary atresia. J. Pediatr. Surg. 16, 284-290.

Anzano, M.A., OLson, J.A., Lamb, A.J. (1980). Morphological alterations in the trachea and the salivary gland following the induction of rapid synchronous vitamin A deficiency in rats. Am. J. Pathol. 98, 717-732.

Aron, H.C.S., Craig, R.M., Farmer, C.J., Kendell, H.W., Schwemlein, G.X. (1946). Effect of elevated body temperature on plasma vitamin A and carotene. Proc. Soc. Exp. Biol. Med. 61, 271-276.

Arroyave, G., Wilson, D., Mendez, J., Behar, M., Scrimshaw, N.S. (1961). Serum and liver vitamin A and lipids in children with severe protein malnutrition. 9, 180-185.

Badley, B.W.O., Mruphy, G.M., Boucher, I.A.D., Sherlock, S., (1970). Diminished micellar phase lipid in patients with chronic non-alcoholic liver disease and steatorrhoea. Gastroent. 58, 781-789.

Baker, A.J., Charlton, C.P.J., Smith, S., Tarlow, M.J. (1988). Salivary caffeine half-life - A steelyard of liver function. Paediatric Res. Soc. (abstract). Jersey (in press).

Baker, H., Frank, O., Feingold, S., Leevy, C.M. (1967). Vitamin distribution in human plasma proteins. Nature. 215, 84-85.

Bates, C.J. (1983). Vitamin A in pregnancy and lactation. Proc. Nutr. Soc. 42, 65-79.

Bates, J., MacClain, C.J. (1981). The effect of severe zinc
deficiency on serum levels of albumin, trasnferrin and pre-
albumin in man. Am. J. Clin. Nutr. 34, 1655-1660.

Barnard, D.L., Heaton, K.W. (1973). Bile acid and vitamin A
absorption in man: the effect of two bile acid binding
agents, cholestyramine and lignin. Gut. 14, 316-318.

Barnes, B.C., Wollaeger, E.E., Mason, H.L. (1950). The
comparative absorption of vitamin A from a water-miscible
and an oily preparation by normal human adults and patients
with steatorrhoea. J. Clin. Investig. 29, 982-987.

Bauerfeind, J.C. (1980). The safe use of vitamin A. A report
of the International Vitamin A Consultative Group (IVACG).
Washington.

Beaver, D.L. (1961). Vitamin A deficiency in the germ free
rat. Am. J. Pathol. 38, 335-350.

Benedich, A., Langseith, L. (1989). Safety of vitamin A. Am.
J. Clin. Nutr. 49, 358-371.

Beverley, D.W., Kelleher, J., MacDonald, A., Littlewood,
J.M., Robinson, T. (1987). Comparison of four pancreatic
extracts in cystic fibrosis. Arch. Dis. Child. 62, 564-568.

Bieri, J.G., Hoeg, J.M., Schaefer, J., Zech, L.A., Bryan
Brewer jn, H. (1984). Vitamin A and vitamin E replacement in
abetalipo-proteinaemia. Ann. Intern. Med. 100, 238-239.

Bieri, J.G., MacDaniel, E.G., Rogers, W.E. (1969). Survival
of germ free rats without vitamin A. Science. 163, 574-575.

Bishara, S., Merin, S., Cooper, M., Azizi, E., Delpre, G.,
Deckelbaum, R.J. (1982). Combined vitamin A and E therapy
prevents retinal electrophysiological deterioration in
abetalipo-proteinaemia. Br. J. Ophthalmol. 66, 767-770.

Blatz, P.E., Estrada, A. (1972). Investigation of the
molecular behaviour of the Carr-Price reaction. Anal. Chem.
44, 570-573.

Blomhoff, R., Helgerud, P., Rasmussen, M., Berg, T., Norum, K.R. (1982). In vivo uptake of chylomicron [^{3}H] retinyl ester by rat liver: evidence for retinol transfer from parenchymal to non-parenchymal cells. Proc. Natl. Acad. Sci. 79, 7326-7330.

Borek, C., Smith, J.E., Soprano, D.R., Goodman, D.S. (1981). Regulation of retinol-binding protein metabolism by glucocorticoid hormones in cultured H_4IIEC_3 liver cells. Endocrinol. 109, 386-391.

Borgstrom, B. (1985). Fat assimilation. In "Bockus Gastroenterology" (Ed Berk, J.E.). 3, pp1510-1519. W.B. Saunders Co, London.

Bosin, E., Monji, N. (1983). Quantitation of serum retinol-binding protein by an enzyme-linked immunosorbant assay. Anal. Biochem. 133, 283-287.

Bowman, W.C., Rand, M.J., (Eds). (1982). The alimentary canal and drugs used in the treatment of its disorders. In "Textbook of Pharmacology". 2nd edition. pp25.1-25.40. Blackwell Scientific Publications, London.

Bridges, C.D.B. (1984). Retinoids in photosensitive systems. In The Retinoids. (eds Sporn, M.B., Roberts, A.B., Goodman, D.S.). 2, pp126-171. Academic Press Inc, London.

Bridges, C.D.B., Alvarez, R.A., Fong, S.L. (1982). Vitamin A in human eyes: amount, distribution and composition. Investig. Ophthalmol. Vis. Sci. 72, 706-714.

Brissot, P., Le Treut, A., Dien, G., Cohengin, M., Simon, M., Bourel, M. (1978). Hypovitaminemia in idiopathic hemochromatosis and hepatic cirrhosis. Digestion. 17, 469-478.

Campos, F.A.C.S., Flores, H., Underwood, B.A. (1987). Effect of an infection on vitamin A status of children as measured by the relative dose response (RDR). Am. J. Clin. Nutr. 46, 91-94.

Carney, E.A., Russell, R.M. (1980). Correlation of dark adaptation test results with serum vitamin A levels in diseased adults. J. Nutr. 110, 552-557.

Chandra, R.K. (1988). Increased bacterial binding to respiratory epithelial cells in vitamin A deficiency. Br. Med. J. 297, 834-835.

Charlton C.P.J., Buchanan, E., Holden, C., Green, A., Booth, I.W., Tarlow, M.J. (1988). Intensive enteral feeding improves nutrition in candidates for liver transplantation. British Paediatric Association (abstract). York.

Chopra, J.G., Kevany, J. (1970). Hypovitaminosis A in the Americas. Am. J. Clin. Nutr. 23, 231-241.

Chytil, F., Ong, D.E. (1979). Cellular retinol and retinoic acid-binding proteins in vitamin A action. Fed. Proc. 38, 2510-2518.

Cogan, U., Kopelman, M., Mokady, S., Shinitzky, M. (1976). Binding affinities of retinol and related compounds to retinol-binding proteins. Eur. J. Biochem. 65, 71-78.

Congden, P.J., Bruce, G., Rothburn, M.M., Clarke, P.C.N., Littlewood, J.M., Kelleher, J., Losowsky, M.S. (1981). Vitamin status in treated patients with cystic fibrosis. Arch. Dis. Child. 56, 708-714.

Constantino, J.P., Kuller, L.H., Begg, L., Redmond, C.K., Bates, M.W. (1988). Serum level changes after administration of a pharmacological dose of beta-carotene. Am. J. Clin. Nutr. 48, 1277-1283.

Corey, J.E., Hayes, K.C. (1972). CSF fluid, growth, hematology in relation to retinol status of the rat in acute vitamin A deficiency. J. Nutr. 102, 1585-1594.

Dawson, P. (1986). The effect of gentamicin therapy on urinary excretion of retinol-binding protein, lysozyme and electrolytes. Thesis for M. Phil, Aston University, Birmingham.

De, N.K. (1937). The assimilation of vitamin A and carotene by rats from some common foods with a note on the conversion factor IU/E proposed by the International Vitamin Conference. Ind. J. Med. Res. 24, 751-766.

Deluca, H.F. (1979). Retinoic acid metabolism. Fed. Proc. 38, 2519-2523.

De Luca, L.M. (1977). The direct involvement of vitamin A in glycosyl transfer reactions of mammalian membranes. Vit. Horm. 35, 1-57.

De Luca, L.M., Little, E.P., Wolf, G. (1969). Vitamin A and protein synthesis by rat intestinal mucosa. J. Biol. Chem. 244, 701-708.

De Sole, G., Belay, Y., Zegeye, B. (1987). Vitamin A deficiency in Southern Ethiopia. Am. J. Clin. Nutr. 45, 780-784.

Dohlman, C.H., Kalevar, V. (1972). Cornea in hypo-vitaminosis A and protein deficiency. Isr. J. Med. Sci. 8, 1179-1183.

Donoghue, S., Donawick, W.J., Kronfeld, D.S. (1983). J. Nutr. 113, 2197-2204.

Draper, H.H. (1980). In Vitamin E. A comprehensive treatise (Ed Machlin, L.J.). 5D, pp272-288. Marcel Decker Inc, New York.

Durie, P.R., Newth, C.J., Forstner, G.G., Gall, D.G. (1980). Malabsorption of MCT in infants with cystic fibrosis: correction with pancreatic enzyme supplements. J.Pediatr. 96, 862-864.

Dutta, S.K., Bustin, M.P., Russell, R.M., Costa, B.S. (1982). Deficiency of fat-soluble vitamins in treated patients with pancreatic insufficiency. Ann. Intern. Med. 97, 549-552.

El-Goreb, M.I., Underwood, B.A. (1973). Solubilisation of beta-carotene and retinol into aqueous solution of mixed micelles. Biochemica et Biophysica. Acta. 306, 58-66.

El-Goreb, M.I., Underwood, B.A., Loerch, J.D. (1975). The role of bile salts in the uptake of beta-carotene and retinol by rat everted gut sacs. Biochimica et Biophysica. Acta 401, 265-277.

Eastham, R.D. (1985). Biochemical values in clinical medicine (Ed Wright). pp423-430. Bristol.

Flores, H., Campos, F., De Araujo, C.R.C., Underwood, B.A. (1984). Assessment of marginal vitamin A deficiency in brazilian children using the relative dose response procedure. Am. J. Clin. Nutr. 40, 1281-1284.

Flores, De Araujo, C.R.C. (1984). Liver levels of retinol in unselected necropsy specimens: a prevalence survey of vitamin A deficiency in Recife, Brazil. Am. J. Clin. Nutr. 40, 146-152.

Forsgren, L. (1969). Studies on the intestinal absorption of labelled fat-soluble vitamins (A, D, E and K) in the absence of bile in man. Acta. Chir. Scand. s 399, 1-29.

Fredrickson, B., Blackberg, L. (1980). Lingual lipase: an important lipase in the digestion of dietary lipids in cystic fibrosis. Pediatr. Res. 14, 1387-1390.

Frolick, C.A., Olson, J.A. (1984). Extraction, separation and chemical analysis of retinoids. In "The Retinoids." (Ed Sporn, M.B., Roberts, A.B., Goodman, D.S.). 1 , pp182-227. Academic Press Inc, London.

Fulton, A.B., Hansen, R.M., Underwood, B.A., Schwachman, H., Barg, D.C. (1982). Scotopic thresholds and plasma retinol in cystic fibrosis. Investig. Ophthalmol. Vis. Sci. 23, 370.

Futterman, S., Andrews, S.J. (1964). The comparison of liver vitamin A ester and the synthesis of vitamin A ester by liver microsomes. J.Biol. Chem. 239, 4077-4080.

Ganguly, J. (1969). Absorption of vitamin A. Am. J. Clin. Nutr. 22, 923-933.

Garry, P.J., Hunt, W.C., Bandrofchak, J.L., Vanderjagt, D., Goodwin, J.S. (1987). Vitamin A intake and plasma retinol levels in healthy elderly men and women. Am. J. Clin. Nutr. 46, 989-994.

Genest, A.A., Sarwono, D., Gyorgy, P. (1967). Vitamin A blood serum levels and electroretinogram in 5-14 year age group in Indonesia and Thailand. Am. J. Clin. Nutr. 20, 1275-1279.

Glover, J., Moxley, L., Muhilal, H., Weston, S. (1974). Micro-method for fluorimetric assay of RBP in blood plasma. Clin. Chim. Acta. 50, 371-380.

Goodchild, M.C. (1986). Practical management of nutrition and gastrointestinal tract in cystic fibrosis. J. Royal Soc. Med. 79, s12, 32-35.

Goodman, D.S. (1981). Retinoid binding proteins in plasma and cells. Ann. N.Y. Acad. Sci. 359, 69-78.

Goodman, D.S. (1984a). Vitamin A and retinoids in health and disease. N.Eng. J. Med. 310, 1023-1031.

Goodman, D.S. (1984b). Plasma retinol-binding protein. In "The Retinoids." (Eds Sporn, M.B., Roberts, A.B., Goodman, D.S.). 2 , pp42-82. Academic Press Inc, London.

Goodman, D.S., Blaner, W.S. (1984). Biosynthesis, absorption and hepatic metabolism of retinol. In "The Retinoids." (Ed Sporn, M.B., Roberts, A,B., Goodman, D.S.).2, 2-34. Academic Press Inc, London.

Goodman, D.S., Blomstrand, R., Werner, B., Huang, H.S., Shiratori, T. (1966). The intestinal absorption and metabolism of vitamin A and carotene in man. J. Clin. Investig. 45, 1615-1623.

Goodman, D.S., Huang, H.S. (1965). Biosynthesis of vitamin A with rat intestinal enzymes. Science (N.Y.). 149, 879-880.

Goodman, D.S., Huang, H.S., Shiratori, T. (1965). Tissue distribution and metabolism of newly absorbed vitamin A in the rat. J. Lipid Res. 6, 390-396.

Hansen, L.G., Warwick, W.J. (1969). A fluorometric micromethod for serum vitamins A and E. Am. J. Clin. Path. 51, 538-541.

Harding, A.E., Muller, D.P.R., Thomas, P.K., Willison, H.J. (1982). Spinocerebellar degeneration secondary to chronic intestinal malabsorption: a vitamin E deficiency syndrome. Ann. Neurol. 12, 419-424.

Harris, R., Norman, A.P., Payne, W.W. (1955). The effect of pancreatin therapy on fat absorption and nitrogen retention in children with fibrocystic disease of the pancreas. Arch. Dis. Child. 30, 424-427.

Harrison, E.H., Smith, J.E., Goodman, D.S. (1979). Unusual properties of retinyl palmitate hydrolase activity in rat liver. J. Lipid Res. 20, 760-771.

Hatchell, D.L., Sommer, A. (1984). Detection of ocular surface abnormalities in experimental vitamin A deficiency. Arch. Ophthalmol. 102, 1389-1393.

Hayes, K.C., Cousins, R.J. (1970). Vitamin A deficiency and bone growth. Calc.Tiss. Res. 6, 120-132.

Helgerud, P., Petersen, L.B., Norum, K.R. (1983). Retinol esterification by microsomes from the mucosa of human small intestine. J. Clin. Investig. 71, 747-753.

Hermon-Dowling, R. (1973). Intestinal adaptation. N.Eng. J. Med. 288, 520-521.

High, E.G. (1969). Some aspects of nutritional vitamin A levels in preschool children of Beaufort County, South Carolina. Am. J. Clin. Nutr. 22, 1129-1132.

Hollander, D., Rubie, P.E. (1978). Beta-carotene intestinal absorption: bile, fatty acid, pH and flow rate effects on transport. Am. J. Physiol. E, 686-691.

Howard, L., Ovesen, L., Satya-Murti, S., Chu, R. (1982). Reversible neurological symptoms caused by vitamin E deficiency in a patient with short bowel syndrome. Am. J. Clin. Nutr. 36, 1243-1249.

Hume, E.M., Krebs, H.A. (1949). Vitamin A requirement of human adults. Med. Res. Council Spec. Rep. Ser. 264, 1-45.

Imes, S., Pinchbeck, B., Dinwoodie, A, Walker, K., Thompson, A.B.R. (1987). Vitamin A status in 137 patients with crohns disease. Digestion. 37, 166-170.

Ingenbleek, Y., Van Den Schrieck, H.G., Dewayer, P., De Visscher, M. (1975). The role of retinol-binding protein in protein-calorie malnutrition. Metabolism. 24, 633-641.

Inua, M., Duggan, M.B., West, C.E., Whittle, H.C., Kogbes, O.I., Sandford-Smith, J.H., Glover, J. (1983). Post-measles corneal ulceration in children in Northern Nigeria: the role of vitamin A, malnutrition and measles. Annal. Tropical. Paediatr. 3, 181-191.

IUPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN). (1982). Nomenclature of retinoids. Recommendations 1981. Eur. J. Biochem. 129, 1-5.

James, H.O., West, C.E., Duggan, M.B., Ngwa, M. (1984). A controlled study on the effect of injected water-miscible retinyl palmitate on plasma concentrations of retinol and retinol-binding protein in children with measles in Northern Nigeria. Acta. Paediatr Scand. 73, 22-28.

Kahan, J. (1969). The vitamin A absorption test. 1. Studies on children and adults without disorders in the alimentary tract. Scan. J. Gastroent. 4, 313-324.

Kahan, J. (1970). The vitamin A absorption test. 2. Studies on children and adults with disorders in the alimentary tract. Scan. J. Gastroent. 5, 5-12.

Kalz, F., Schafer, A. (1958). Vitamin A serum levels after ingestion of different vitamin A preparations. Can. Med. Assoc. J. 79, 918-919.

Kanai, M., Raz, A., Goodman, D.S. (1968). Retinol-binding protein: the transport protein for vitamin A in human plasma. J.Clin. Investig. 47, 2025-2044.

Kark, J.D., Smith, A.H., Hames, C.G. (1982). Serum retinol and the inverse relationship between serum cholesterol and cancer. Br. Med. J. 284, 152-154.

Kaser, M., Stekol, J.A. (1942). A clinical study of the Carr-Price reaction for the determination of beta-carotene and vitamin A in biological materials. J. Lab. Clin. Med. 28, 904-909.

Kaufman, S.S., Murray, N.D., Wood, R.P., Shaw, B.W., Vanderhook, J.A. (1987). Nutritional support for the infant with extrahepatic biliary atresia. J.Pediatr. 110, 679-686.

Kelleher, J. (1987). Laboratory measurement of nutrition in cystic fibrosis. J. Royal. Soc. Med. 80 s15, 25-29.

Kelleher, J., Walters, M.P., Johnson, A.W. (1988). In vivo and in vitro studies of microsphere pancreatic supplements. J. Pediatr. Gastroenterol. Nutr. 7 s1, s22-s29.

Kobayashi, A., Kawai, S., Ohbe, Y., Benno, Y. (1988). Fecal flora of infants with biliary atresia: effects of the absence of bile on fecal flora. Am. J. Clin. Nutr. 48, 1211.

Kusin, J.A., Reddy, V., Sivakumar, B. (1974). Vitamin E supplements and the absorption of a massive dose of vitamin A. Am. J. Clin. Nutr. 27, 774-776.

Large, S., Neal, G., Glover, J., Thanangeul, O., Olson, R.E. (1980). The early changes in RBP and pre-albumin concentration in plasma of protein-energy malnourished children, after treatment with retinol and an improved diet. Br. J. Nutr. 43, 393-402.

Lentner, C. (ed) (1984). Physical chemistry, composition of blood, haematology, somatometric data. In Geigy Scientific Tables. 3, pp125-134.

Lilly, J.R., Javitt, N.B. (1976). Biliary lipid excretion after hepatic portoenterostomy. Annal. Surg. 184, 369-375.

Lippe, B., Hensen, L., Mendoza, G., Finerman, M., Welch, M. (1981). Chronic vitamin A intoxication. Am. J. Dis. Child. 135, 634-636.

Littlewood, J.M. (1986). An overview of the management of cysttic fibrosis. J. Royal Soc. Med. 79 s12, 55-63.

-312-

Littlewood, J.M., Congden, P.J., Bruce, G., Kelleher, J., Rothburn, M., Losowsky, M.S., Clarke, P.C.N. (1980). Vitamin A status in treated cystic fibrosis. In "Perspectives in cystic fibrosis" (ed Sturgess, J.M.) pp166-171. Canadian cystic fibrosis foundation, Toronto.

Littlewood, J.M., MacDonald, A. (1987). Rationale of modern dietary recommendations in cystic fibrosis. J. Royal Soc. Med. s15, 16-24.

Loerch, J.D., Underwood, B.A., Lewis, K.C. (1979). Response of plasma levels of vitamin A to a dose of vitamin A as an indicator of hepatic vitamin A reserves in rats. J. Nutr. 109, 778-786.

Logan, W.S. (1972). Vitamin A and keratinisation. Arch. Derm. 105, 748-753.

Losowsky, M.S., Walker, B.E. (1969). Liver disease and malabsorption. Gastroent. 56, 589-600.

MacLaren, D.S. (1981). The luxus vitamins - A and B_{12}. Am. J. Clin. Nutr. 34, 1611-1616.

Main, A.N.H., Mills, P.R., Russell, R.I., Bronte-Stewart, J., Nelson, L.M., MacLelland, A., Shenkin, A. (1983). Vitamin A deficiency in crohn's disease. Gut. 24, 1169-1175.

Matoltsy, A.G. (1976). Keratinisation. J. Investig Derm. 67, 20-25.

Martini, G.A., Phear, E.A., Roebner, B., Sherlock, S. (1957). The bacterial content of the small intestine in normal and cirrhotic subjects: relation to methionine toxicity. Clin. Sci. 16, 35-51.

Mearns, M.B. (1985). Personal practise: cystic fibrosis. Arch. Dis. Child. 60, 272-277.

Meguid, M.M., Landel, A.M., Oey, L., MacLaren, D.S. (1988). Plasma carotenoid profiles in normals and patients with cancer. J. Parent. Ent. Nutr. 12, 147-151.

Mendez, J., Scrimshaw, N.S., Salvado, C., Selva, M.L. (1959). Appl. Physiol. 14, 768-770.

Mezey, E. (1978). Liver disease and nutrition. Gastroent. 74, 770-783.

Milton, R.C., Reddy, V., Naidu, A.N. (1987). Mild vitamin A deficiency and childhood morbidity - an Indian experience. Am. J. Clin. Nutr. 46, 827-829.

Mobarhan, S., Russell, R.M., Underwood, B.A., Wallingford, J., Mathieson, R.D., Al-Midani, H. (1981). Evaluation of the relative dose response test for vitamin A nutriture in cirrhotics. Am. J. Clin. Nutr. 34, 2264-2270.

Moeller, D.D., Dun, G.D., Klotz, A.P. (1974). Pancreatic function in malabsorbing alcoholic cirrhotics. Am. J. Dig. Dis. 19, 799-784.

Moody, B.J. (1982). Changes in serum concentrations of thyroxine-binding pre-albumin and retinol-binding protein following burn injury. Clinica. Chimica. Acta. 118, 87-92.

Moore, T. (1957). Vitamin A. Elsevier Publishing Co., London.

Morita, A., Nakano, K. (1982). Effect of chronic immobilisation stress on tissue distribution of vitamin A in rats fed on a diet with adequate vitamin A. J. Nutr. 112, 789-795.

Mowat, A.P. (1987). Extra-hepatic biliary atresia. In Liver disorders in children. 5, pp78-93. Butterworth and Co., London.

Muller, D.P.R. (1986). Vitamin E - it's role in neurological function. Postgrad. Med. J. 62, 107-112.

Murray, T.K., Grice, H.C. (1961). Absorption of vitamin A after thoracic duct ligation in the rat. Can. J. Biochem. Biophys. 39, 1103-1106.

Muto, Y., Goodman, D.S. (1972). Vitamin A transport in rat plasma. Isolation and characterisation of RBP. J. Biol. Chem. 247, 2533-2541.

Muto, Y., Goodman, D.S., Smith, J.E., Milch, P.O., Goodman, D.S. (1972). Vitamin A transport in rat plasma. Isolation and characterisation of RBP. J.Biol. Chem. 247, 2533-2541.

Navab, M., Smith, J.E., Goodman, D.S. (1977). Rat plasma pre-albumin. Metabolic studies on effects of vitamin A status and tissue distribution. J. Biol. Chem. 252, 5107-5114.

Neeld, J.B., Pearson, W.N. (1963). Macro- and micro-methods for the determination of serum vitamin A using trifluoroacetic acid. J. Nutr. 79, 454-462.

Nelson, J.D., Havener, V.R., Cameron, J.D. (1983). Cellulose acetate inpressions of the ocular surface. Dry eye states. Arch. Ophthalmol. 101, 1869-1872.

Nyberg, A., Berne, B., Nordlinder, H., Busch, C., Eriksson, U., Loof, L., Vahlquist, A. Impaired release of vitamin A from liver in primary biliary cirrhosis. Hepatol. 8, 138-141.

Olson, J.A. (1964). The effect of bile and bile salts on the uptake and cleavage of beta-carotene into retinol ester (vitamin A ester) by intestinal slices. J. Lipid. Res. 5, 402-408.

Olson, J.A. (1967). The metabolism of vitamin A. Pharmacol. Rev. 19, 559-590.

Olson, J.A. (1972). The biological role of vitamin A in maintaining epithelial tissues. Isr. J. Med. Sci. 8, 1170-1178.

Olson, J.A. (1982). New approaches to methods for the assessment of nutritional status of the individual. Am. J. Clin. Nutr. 35, 116-1168.

Olson, J.A. (1987). Recommended dietary intakes (RDI) of vitamin A in humans. Am. J. Clin. Nutr. 45, 704-716.

Olson, J.A., Gunning, D.B. (1983). The storage form of vitamin A in rat liver cells. J. Nutr. 113, 2184-2191.

Olson, J.A., Gunning, D.B., Tilton, R.A. (1984). Liver concentrations of vitamin A and carotenoids as a function of age and other parameters, of American children who died of various causes. Am. J. Clin. Nutr. 39, 903-910.

O'Neil, R.M., Johnson, O.C., Schaefer, A.E. (1970). Guidelines for classification and interpretation of group blood and urine data collected as part of the national nutritional survey. Pediatr. Res. 4, 103-106.

Palin, D., Underwood, B.A., Denning, C.R. (1979). The effect of oral zinc supplementation on plasma levels of vitamin A and retinol-binding protein in cystic fibrosis. Am. J. Clin. Nutr. 32, 1253-1259.

Parkinson, C.E., Gal, I. (1972). Factors affecting the laboratory management of human serum and liver vitamin A analysis. Clin. Chimica. Acta. 40, 83-90.

Passmore, R., Eastwood, M.A. (1986). Fat-soluble vitamins. In "Human nutrition and dietetics." (ed Passmore, R., Davidson) 8th edition. 13, 132-145. Church & Livingstone, London.

Persson, B., Tunell, R., Ekengren, K. (1965). Chronic vitamin A intoxication during the first half year of life. Acta. Paed. Scand. 54, 49-60.

Peterson, P.A. (1971). Demonstration in serum of two physiological forms of the human retinol-binding protein. Eur. J. Clin. Investig. 1, 437-447.

Peterson, P.A., Rask, L., Ostberg, L., Anderson, L., Kamweno, F., Pertoft, H. (1973). Studies on the transport and cellular distribution of vitamin A in normal and vitamin A deficient rats with special reference to the vitamin A binding plasma protein. J.Biol. Chem. 284, 4009-4022.

Raica, N., Scott, J., Lowry, L., Sauberlich, H.E. (1972). Vitamin A concentration in human tissues collected from five areas in the United States. Am. J. Clin. Nutr. 25, 291-296.

Rao, V., Friend, J., Thoft, R.A., Underwood, B.A., Reddy, P.R. (1987). Conjunctival goblet cells and mitotic rate in children with retinol deficiency and measles. Arch. Ophthalmol. 105, 378-380.

Raynor, R.J., Tyrrell, J.C., Hiller, E.J., Marenah, C., Neugebauer M., Vernon, S., Brimlow, G. (1988). Vitamin A deficiency in cystic fibrosis. British Paediatric Association (abstract), York.

Redgrave, T. (1970). Formation of cholesteryl estr rich particulate lipid during metabolism of chylomicrons. J. Clin. Investig. 49, 465-471.

Robinson, W.G., Kuwabara, T., Bieri, J.G. (1980). Deficiencies of vitamins E and A in the rat. Retinal damage and lipofuscin accumulation. Investig. Ophthalmol. 19, 1030-1037.

Robrigues, M.S., Irwin, M.I. (1972). A conspectus of research on vitamin A requirements of man. J. Nutr. 102, 909-968.

Russell, R.M. (1980). Vitamin A and zinc metabolism in alcoholism. Am. J. Clin. Nutr. 33, 2741-2749.

Russell, R.M., Iber, F.L., Bustin, M., Goldberg, N., Miller, P., Krasinski, S. (1982). Failure of the relative dose response test to predict vitamin A deficiency in patients with protien calorie malnutrition. Am. J. Clin. Nutr. 35, 857.

Russell, R.M., Morrison., S.A., Smith, F.R., Oaks, E.V., Carney, E.A. (1978). Vitamin A reversal of abnormal dark adaptation in cirrhosis. Annal. Intern. Med. 88, 622-626.

Russell, R.M., Smith, V.C., Multack, R., Krill, A.E., Rosenberg, I.H. (1973). Dark adaptation testing for diagnosis of subclinical deficiency and evaluation of therapy. Lancet. 2, 1161-1164.

Sanderford-Smith, J. (1988). Children's eye conditions in developing countries. Annales. Nestle. 46, 27-37.

Sauberlich, H.E., Hodges, R.E., Wallace, D.L., Kolder, H., Canham, J.E., Hood, J., Raica, N., Lowry, L.K. (1974). Vitamin A metabolism and requirements in teh human studied with the use of labelled retinol. Vit. Horm. 32, 251-275.

Shepherd, A.N., Bedford, G.J., Hill, A., Bouchier, I.A.D. (1984). Primary biliary cirrhosis, dark adaptometry, electro-oculography and vitamin A state. Br. Med. J. 289, 1485-1486.

Sivakumar, B., Reddy, V. (1972). Absorption of labelled vitamin A in children during infection. Br.J. Nutr. 27, 299-304.

Skirantia, S.G., Reddy, V. (1970). Effect of a single massive dose of vitamin A on serum and liver levels of the vitamin. Am. J. Clin. Nutr. 23, 114-118.

Skrede, S., Blomhoff, J.P., Elgjo, K., Gjone, E. (1975). Serum proteins in diseases of the liver. Scan. J. Clin. Investig. 35, 399-406.

Smith, F.R., Goodman, D.S. (1971). The effects of diseases of the liver, thyroid and kidneys on the transport of vitamin A in human plasma. J. Clin. Investig. 50, 2426-2436.

Smith, F.R., Goodman, D.S. (1976). Vitamin A transport in human vitamin A toxicity. N. Eng. J. Med. 294, 805-808.

Smith, F.R., Goodman, D.S., Zaklama, M.S., Gabr, M.K., Maraghy, S.E., Patwardhan, V.N. (1973a). Serum vitamin A, retinol-binding protein and pre-albumin concentrations in protein calorie malnutrition. 1. A functional defect in hepatic retinol release. Am. J. Clin. Nutr. 26, 973-981.

Smith, F.R., Lindenbaum, J. (1974). Human serum retinol transport in malabsorption. Am. J. Clin. Nutr. 27, 700-705.

Smith, F.R., Underwood, B.A., Denning, C.R., Varma, A., Goodman, D.S. (1972). Depressed plasma retinol-binding protein levels in cystic fibrosis. J. Lab. Clin. Med. 80, 423-433.

Smith, J.E., Borek, C., Goodman, D.S. (1978). Regulation of retinol-binding protein metabolism in cultured rat liver cell lines. Cell. 1978, 865-873.

Smith, J.E., Brown, E.D., MacDaniel, E.G., Chan, W. (1976). Alterations in vitamin A metabolism during zinc deficiency and food and growth restrictions. J. Nutr. 106, 569-574.

Smith, J.E., Goodman, D.S. (1979). Retinol-binding protein and the regulation of vitamin A transport. Fed. Proc. 38, 2504-2509.

Smith, J.E., MacDaniel, E.G., Fan, F.F., Halsted, J.A. (1973b). Zinc: a trace element essential in vitamin A metabolism. Science (N.Y.). 181, 954-955.

Smith, J.E., Muto, Y., Goodman, D.S. (1975). Tissue distribution and subcellular localisation of retinol binding protein in normal and vitamin A deficient rats. J. Lipid. Res. 16, 318-323.

Sokol, R.J. (1987). Assessing vitamin E status in childhood cholestasis. J. Pediatr. Gastroent. Nutr. 6, 10-13.

Sommer, A., Green, W.R. (1982). Goblet cell response to vitamin A treatment for corneal xerophthalmia. Am. J. Ophthalmol. 94, 213-215.

Soprano, D.R., Smith, J.E., Goodman, D.S. (1982). Effect of retinol status on RBP biosynthesis rate and translatable messenger RNA level in rat liver. J. Biol. Chem. 257, 7693-7697.

Sporn, M.B., Roberts, A.B. (1983). Role of retinoids in the differentiation and carcinogenesis. Cancer Res. 43, 3034-3040.

Stewart Trusnell, A. (1985). ABC of nutrition. Vitamins I. Br. Med. J. 291, 1033-1035.

Stirling, H.F., Laing, S.C., Barr, D.G.B. (1986). Hypercarotenaemia and vitamin A overdosage from proprietary baby food. Lancet. 1, 1089.

-319-

Sullivan, W.R., MacCulley, J.P., Dohlman, C.H. (1973). Return of goblet cells after vitamin A therapy in xerosis of the conjunctiva. Am. J. Ophthalmol. 75, 720-725.

Swaminathan, M.C., Susheela, T.P., Thimmayamma, B.V.S. (1970). Field prophylactic trial with a single annual massive dose of vitamin A. Am. J. Clin. Nutr. 23, 119-122.

Takahashi, Y.I., Smith, J.E., Winick, M. Goodman, D.S. (1975). Vitamin A deficiency and fetal growth and development in the rat. J. Nutr. 105, 1299-1310.

Thoft, R.A., Friend, J., Kinoshita, S., Nikolie, L., Foster, C.S. (1984). Ocular acatricial pemphigoid associated with hyperproliferation of conjunctival epithelium. Am. J. Ophthalmol. 98, 37-42.

Thompson, S.Y. (1964). Factors affecting the absorption of carotene and its conversion to vitamin A. Exp. Eye. Res. 3, 392-405.

Thompson, J.N., MacHowell, J., Pitt, G.A. (1964). Vitamin A and reproduction in rats. Proc. Royal Soc. London. 159, 510-535.

Thurnham, D.I., Smith, E. Flora, P.S. (1988). Liquid chromatographic concurrent assay of retinol, alpha-tocopherol, beta-carotene, alpha-carotene, lycopene and beta-cryptoxanthin in plasma with tocopherol acetate as an internal standard. Clin. Chima. 34, 377-381.

Underwood, B.A. (1978). Hypovitaminsis A and it's control. Bull. W.H.O. 56, 521-541.

Underwood, B.A. (1984). Vitamin A and human nutrition. In The Retinoids. (eds Sporn, M.B., Roberts, A.B., Goodman, D.S.). 1, pp282-377. Academic Press Inc, London.

Underwood, B.A., Denning, C.R. (1972). Blood and liver concentrations of vitamins A and E in children with cystic fibrosis. Pediatr. Res. 6,26-31.

Vahlquist, A., Rask, L., Peterson, P.A., Berg, T. (1975). The concentration of retinol-binding protein, pre-albumin and transferrin in the sera of newly delivered mothers and children of various ages. Scan. J. Clin. Lab. Investig. 35, 569-575.

Vahlquist, A., Sjolund, K., Norden, A., Peterson, P.A., Stigmar, G., Johansson, B. (1978). Plasma vitamin A transport and visual dark adaptation in diseases of the intestine and liver. Scan. J. Clin. Lab. Investig. 38, 301-308.

Varley, H., Gowenlock, A.H., Bell, M. (1976). Hormones, vitamins, drugs and poisons. In "Practical clinical biochemistry." 5th edition. 2, 215-223. Heinemann Medical Books, London.

Venkataswamy, G., Glover, J., Cobby, M., Pirie, A. (1977). Retinol-binding protein in serum of xerophthalmic, malnourished children before and after treatment at a nutrition centre. Am. J. Clin. Nutr. 30, 1968-1973.

Wald, G. (1960). The visual function of the vitamins A. Vit. Horm. 18, 417-430.

Walt, R.P., Kemp, C.M., Lyness, L., Bird, A.C., Sherlock, S. (1984). Vitamin A treatment for night blindness in primary biliary cirrhosis. Br. Med. J. 288, 1030-1031.

Weber, F. (1983). Biochemical mechanisms of vitamin A action. Proc. Nutr. Soc. 42, 31-41.

Weber, A.M., Roy, C.C. (1972). The malabsorption associated with chronic liver disease in children. Pediatr. 50, 73-83.

Weber, A.M., Roy, C.C., Morin, C.L., La Salle, R. (1973). Malabsorption of bile acids in children with cystic fibrosis. N. Eng. J. Med. 289, 1001-1005.

Weser, E., Urban, E. (1985). The short bowel syndrome. In "Bockus Gastroenterology." (ed Berk, J.E.). 4th edition. 3, 1792-1802. W.B. Saunders Co, London.

West, K.P., Djunaedi, E., Pandj, A., Kusdiono, Tarwotjo, I., Sommer, A. (1988). Vitamin A supplementation and growth: a randomised trial. Am. J. Clin. Nutr. 48, 1257-1264.

W.H.O.   (1967).   "Requirements of vitamin A, thiamine, riboflavin and niacin." Report of the F.A.O/W.H.O. expert group. Rome (1965). W.H.O. technical report series No 362/F.A.O. nutrition meeting report series No 41. W.H.O, Geneva.

W.H.O.   (1976). "Vitamin A deficiency and xerophthalmia." A report   of   a   joint   W.H.O./U.S.A.I.D.   Meeting. W.H.O. technical series No 590. W.H.O., Geneva.

W.H.O.   (1982).   "Control of vitamin A deficiency and xerophthalmia." W.H.O. technical series No 672. W.H.O., Geneva.

Willett, W.C., Stampfer, M.J., Underwood, B.A., Taylor, J.O., Hennekens, C.H. (1983). Vitamins A, E and carotene: effects odf supplementation on their plasma levels. Am. J. Clin. Nutr. 39, 559-566.

Wolbach, S.B., Howe, P.R. (1925). Tissue changes following deprivation of fat-soluble vitamin A. J.Exp. Med. 42, 753-777.

Woolf, G.M., Miller, C., Kurian, R., Jeejeebhoy, K.N. (1983). Diet for patients with a short bowel: high fat or high carbohydrate. Gastroent. 84, 823-828.

Yang, N.Y.J., Desai, I.D. (1977). Effects of high levels of dietary vitamin E on liver and plasma lipids and fat-soluble vitamins in rats. Am. J. Clin. Nutr. 27, 774-776.

Yeung,   D.L., Veen-Baigent, M.J. (1972). Absorption of retinol and retinyl esters via lymph and the portal vein in the rat. Can. J. Physiol. Pharmacol. 50, 753-760.

Ziegler, R.G., Wilcox, H.B., Mason, T.G., Bill, J.S., Virgo, P.W. (1987). Seasonal variation in intake of carotenoids and vegetables and fruit among white men in New Jersey. Am. J. Clin. Nutr. 45,107-114.

APPENDICES

Appendix i

Abstract presented to the British Paediatric Association in York, April 1988.

Vitamin A supplementation in chronic liver disease.

Bouchard, C.A., Charlton, C.P.J., Baker, A.B., Tarlow, M.J., Marsters, J.B. Liver Unit, Birmingham Children's Hospital. Institute of Child Health, University of Birmingham. Birmingham & Midlands Eye Hospital.

Conventionally, children with chronic liver disease are supplemented with a standard daily dose of 2,500iu of vitamin A as Ketovite (Paines & Byrne). Recently, it has been recommended that supplemented children should achieve serum levels of $1.4-1.7 \mu mol/l$ to maintain adequate retinal delivery without toxicity (1). We measured serum vitamin A levels using a colourimetric method in ten supplemented children (five with extra-hepatic biliary atresia, three with cirrhosis, two with cystic fibrosis and cirrhosis), six of whom had elevated bilirubin levels (mean 204 $\mu mol/l$, range 43-343 $\mu mol/l$). The vitamin A levels ranged from 0.51-1.45 $\mu mol/l$ (mean 0.90 $\mu mol/l$), with only one patient within the desired range.

In conclusion, our previous standard supplementation was found to be insufficient in nine of the ten patients. These patients will required increased doses, with monitoring to achieve the desired serum levels. The priority in chronic liver disease should be to prevent clinical consequences of vitamin A deficiency without causing toxicity.

(1) Kaufman, S.S., Murray, N.D., Wood, R.P., Shaw, B.W., Vanderhoof, J.A. 1987. Nutritional support for the infant with extra-hepatic biliary atresia. J. Paediatr. 110, 679-686.

Appendix ii.

Normal Serum Biochemical values in children.

Carotenoids		0.70-1.70µmol/l
Cholesterol	neonate	1.5-4.0mmol/l
	< 0.5 years	2.0-5.5mmol/l
	0.5-14 years	2.5-6.5mmol/l
Vitamin A		1.00-2.30µmol/l
Vitamin E		9.50-36.40µmol/l
Zinc		10-25µmol/l
Albumin	neonate	25-45g/l
	child	30-50g/l
Alanine transaminase (ALT)	neonate	< 120u/l
	child	< 40u/l
Aspartate transaminase (AST)	neonate	< 100u/l
	child	< 50u/l
Alkaline phosphatase (AP)	neonate	150-600u/l
	child	250-1000u/l
Bilirubin	neonate	< 220µmol/l
	child	< 15µmol/l
Gamma glutamyltransferase (gamma-GT)		
	neonate	< 200u/l
	< 1 year	< 150u/l
	> 1 year	< 30u/l
Partial thromboplastin time (PTT)		< 6-7 secs[*]
Prothrombin time (PT)		< 2-3 secs[*]

[*] compared with the control.