STUDIES OF VITAMIN A IN NUTRITION AND CANCER

by

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To those whom I love, my mother, my sisters and brothers and in memory of my beloved father.

ABBREVIATIONS

HPLC	High pressure liquid chromatography
AUFS	Absorbance units full scale
BHA	Butylhydroxyanisole
PA	Prealbumin
IVN	Intravenous Nutrition
4NQO	4-Nitroquinoline-N-oxide
CRBP	Cellular retinol-binding protein
CRABP	Cellular retinoic acid-binding protein
RBP	Retinol-binding protein
LDL	Low density lipoprotein
HDL	High density lipoprotein
TFA	Trifluoroacetic acid
$SbCL_3$	Antimonytrichloride
NaN_3	Sodium azide
K'	capacity ratio
R_s	Resolution
IS	Internal Standard
R_F	Ratio of the distance travelled by the solute to the distance travelled by solvent front
H:THF:Fac	Hexane:tetrahydrofuran:formic acid
EDTA	Ethylene diamine tetracetic acid
RID	Radial immunodiffusion
PEG	Polyethylene glycol
CRP	C-reactive protein
TG	Triglyceride
TPN	Total parenteral nutrition
-Fe	Iron-deficiency
+Fe	Iron-sufficient
IU	International Units

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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DECLARATION

All the work presented in this thesis was performed by the author, except where otherwise acknowledged.

Signature ....

(Maysoon Al-Saleh)

SUMMARY

This thesis describes the development of specific assays for separation and extraction of vitamin A (retinol) and its metabolites, retinal, retinoic acid, retinol acetate, retinol palmitate and its pro-vitamin carotene. The assay employed a liquid/liquid extraction procedure which gave reproducible recoveries for retinol and its metabolites. This was followed by a high pressure liquid chromatography (HPLC) procedure using a silica column with hexane:tetrahydrofuran:formic acid as mobile phase. This gave excellent chromatographic efficiency and resolution of metabolites and resulted in a highly sensitive and specific assay for all the compounds analysed. The assay was used for serum, and was modified to be applicable also for liver and nutritional solutions. A major benefit of this assay compared with previous methods is that all retinol metabolites can be separated on a single column without requirement for expensive gradient systems.

The HPLC method was compared with the more commonly used fluorimetric method. Fluorimetry provided a more rapid result but was found to be affected by triglyceride and other interfering substances. HPLC was therefore a more accurate assay for patients likely to be receiving nutrients or drugs which would interfere with the fluorimetric method.

A number of studies were performed on vitamin A status in man and rats.

1) Retinol is the only retinoid found in measurable amounts in serum of normal man and rats and those with certain types of tumour and disease.

2) In all studies in man, serum retinol correlated well with its carrier protein, retinol-binding protein (RBP).

3) There is a significant fall in serum retinol concentration during the day in normal individuals.

4) The effect of intravenous nutrition (IVN) on serum retinol was studied. Retinol palmitate was found to be unstable in fat emulsion, but it can be protected from degradation if stored in the dark at room temperature for 24 hours or at 4°C for 7 days. Infusion of 750 µg retinol palmitate intravenously in Intralipid leads to no change in serum retinol, and the retinol palmitate is cleared from the serum within 4 hours. Oral retinol palmitate (160 mg) was also rapidly cleared within 8 hours, and with no effect on serum retinol. The main effect on serum retinol concentration in patients receiving IVN was the presence of an acute phase reaction rather than the dose of retinol provided. In the absence of an acute phase reaction (eg long term IVN) serum retinol was normal or increased to normal in patients receiving IVN. In such patients, this reflects improved protein status as well as adequate vitamin A provision.

5) Rats fed on a vitamin A deficient diet developed low concentrations in liver after 2 weeks, and in serum after 5 weeks. The rapidity of this response may relate to the rate of growth of the rats. Signs of vitamin A deficiency were not seen up to 8 weeks of vitamin A free diet.

6) Serum retinol does not correlate with liver stores of retinol palmitate in either man or rats except in severe deficiency. Assessment of overall retinol status therefore requires liver analysis.

7) The effects of various neoplastic diseases on retinol status in man were studied.

a) There was no relationship between the serum retinol concentration and the presence of oral cancer or leukoplakia. Oral Crohn's Disease did however lead to low serum retinol concentrations.

b) There were no significant differences in serum retinol or liver retinol palmitate between patients with colorectal or gastric cancer and those with benign disease. The presence of metastases at the time of surgery had no effect on serum or liver vitamin A.

c) In patients more than one month post operation for colorectal cancer, there was no significant difference in serum retinol or cholesterol from controls.

These studies have therefore failed to show any association of serum or liver vitamin A with colorectal or oral cancer.

8) The effect of the oral carcinogen (4-nitroquinoline-N-oxide) was studied in rats. Rats treated for 32 weeks developed oral tumours and had low serum and undetectable liver vitamin A. Rats treated for 8 weeks or 14 weeks had not developed tumour but atypia scores were increased, and serum and liver retinoids were very low. The amount of the fall in serum retinol correlated with the rate of increase in weight of the rat. These results suggest that there is increased requirement for retinol during the early stages in development of oral tumours in rats.

9) Iron deficiency led to earlier development of oral tumour in rats with oral carcinogen, but this had no effect on serum or liver vitamin A.

Taken overall, these studies indicate that there is a complex relationship between vitamin A status, protein status, neoplastic disease and the presence of an acute phase reaction. Interpretation of serum retinol results in relation to aetiology of disease or effect of treatment requires careful consideration of these factors.

1) INTRODUCTION

1.1) History

The first evidence for the existence of Vitamin A came when Hopkins (1906 and 1912) showed that a diet of natural fats and oils fed to young rats led to good growth, whereas other fats fed to similar rats did not. His experiments suggested the existence of an "Accessory Food Factor". Osborne and Mendel (1913) and McCollum and Davis (1913) extracted the same growth factor from butter, egg yolk and cod-liver oil with ether, showing that it was fat soluble. McCollum and Davis (1913) came up with the name "fat soluble A" to distinguish it from "water soluble B" which they had found in whey, yeast and rice polishings.

McCollum and Simmonds (1917) found that this "fat soluble A" factor was capable of preventing xerophthalmia. Fridericia and Holm (1925) showed it also prevented night blindness. In 1920 Drummond named the active lipid "vitamin A".

Mellanby (1918), distinguished vitamin A from fat-soluble vitamin D since it was ineffective in the cure of rickets. Rosenheim and Drummond (1920) showed the first relationship between vitamin A and the plant pigment carotene, which was extracted by Steenbock et al (1921) and found to be a growth-promoting substance in plants. When the structure of beta-carotene and that of retinol was elucidated by Karrer et al (1930, 1931 and 1933), the pro-vitamin role of beta-carotene became obvious.

1.2) Chemistry of vitamin A

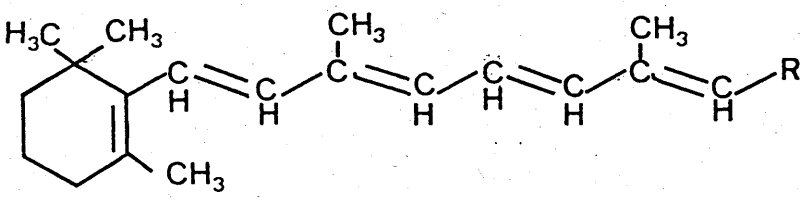
The chemistry of retinol and its derivatives have been reviewed (Isler et al 1967, 1970). Vitamin A is a fat soluble, long chain alcohol which exists in a number of isomeric forms. The most active form and that most usually found in mammalian tissues is the all-trans vitamin A (stereoisomer).

Isomers with cis configuration at the 11 or 13 position occur less commonly and have somewhat lower biological activity. The alcohol form is known as retinol, consisting of a hydrocarbon chain with a beta-ionone ring at one end and an alcohol group at the other (Figure 1).

The terminal alcohol group can be oxidised in the body to an aldehyde (retinal) or a carboxylic acid group (retinoic acid).

Beta-carotene is the only carotenoid which has a structure identical with retinol in both halves of the molecule (Figure 2).

Retinol itself is a pale yellow crystalline substance, soluble in fat and fat solvents, but not in water. Naturally-occurring vitamin A is found only in the animal kingdom, although pro-vitamin A (carotenoids) occurs in the vegetable kingdom. Retinol is often found in an esterified form and esters such as the acetate and palmitate are sometimes preferred for nutritional and medicinal use. Vitamin A, especially the free alcohol, is sensitive to oxygen, acids and ultraviolet light. Vitamin A_2 is closely related to Vitamin A_1, but contains an additional double bond in the beta-ionone ring (3-dehydroretinol). It has about



	R
RETINOL	CH_2OH
RETINAL	CHO
RETINOIC ACID	COOH

Figure 1: Structure of vitamin A and its derivatives

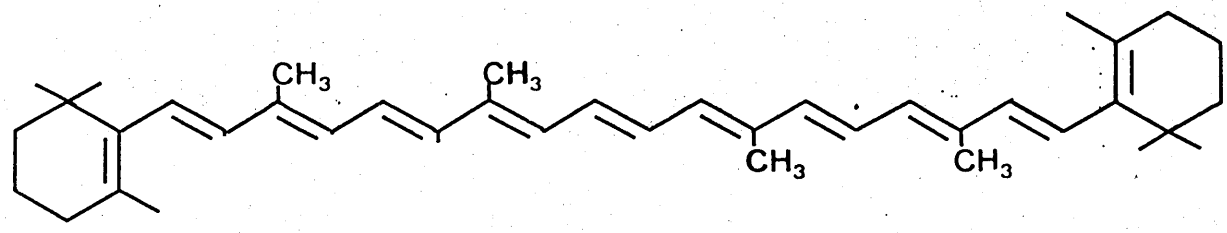


Figure 2: Structure of beta-carotene

half the biological activity of retinol, but is of little biological importance. It occurs with vitamin A in fish liver oil (Davidson et al. 1979).

One international unit of vitamin A is defined as 0.3 µg of all-trans retinol. Generally 1 µg of retinol is assumed to be biologically equivalent to about 6 ug of beta-carotene, or about 12 µg of mixed diet carotenoids (Recommended Dietary Allowances, 1980).

1.3) Absorption

Huang and Goodman (1965) studied the absorption of retinol from the diet, which was present in the form of long-chain fatty acid esters of retinol. The steps of the absorption process begin in the intestine, where hydrolysis of retinyl esters take place, and the resulting retinol is then absorbed into the mucosal cell. Goodman and Olson (1969) proved that dietary carotene was converted to retinol primarily in the intestinal mucosa. In the mucosal cell retinol is re-esterified with long-chain, mainly saturated fatty acids and incorporated into chylomicrons. The chylomicrons are absorbed in the lymph and enter the circulation. All the chylomicrons and retinyl esters are removed from circulation by the liver. (Figure 3).

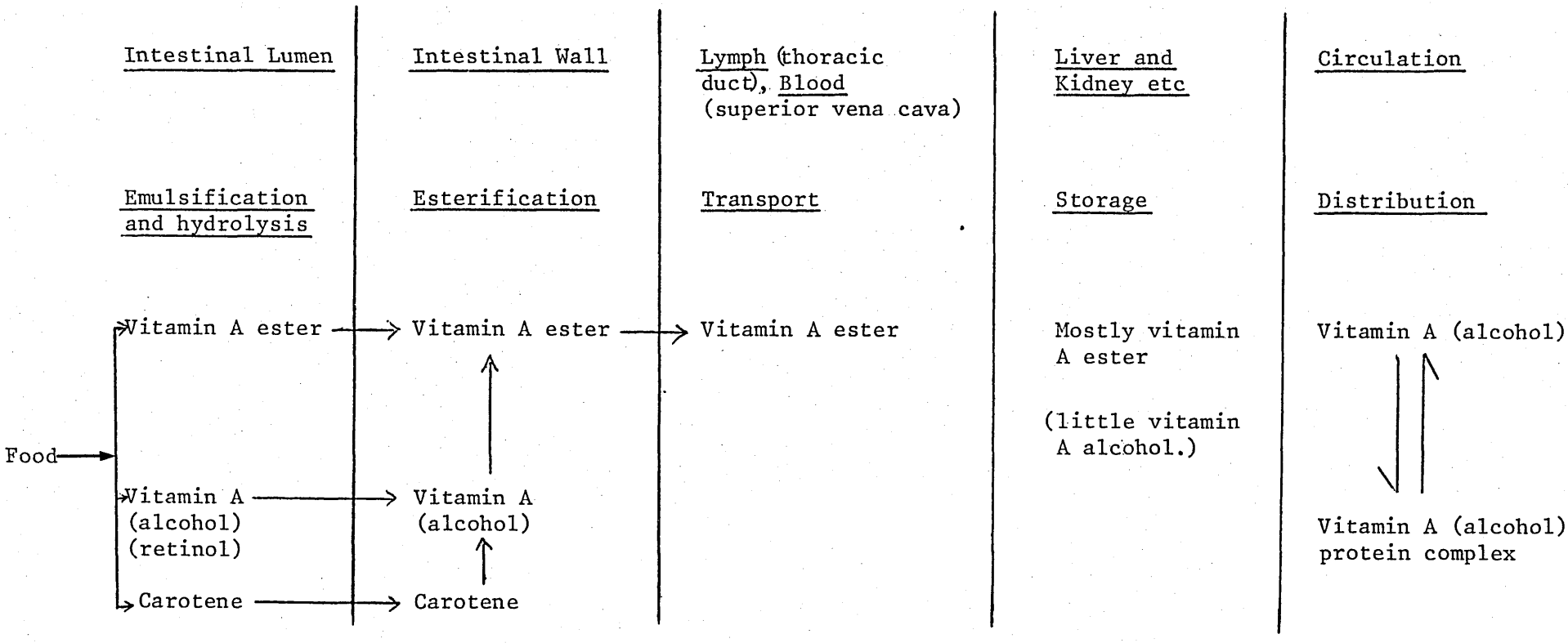


Figure 3: Absorption, storage and transportation of vitamin A (Mark, 1968).

1.4) Transport of Retinol

Kanai et al (1968), studied the transport of retinol. They showed it is carried in plasma, bound to a specific transport protein, retinol-binding protein (RBP). This protein is a single poly-peptide chain with a molecular weight of about 21,000-22,000. Kanai et al (1968) later described this relationship and the role of RBP to retinol. Retinol binds to the apoprotein on a 1:1 molar proportion and the holo-protein circulates in plasma on a 1:1 molar basis with prealbumin (PA). According to Raz et al (1970) their data strongly suggested that RBP has a single binding site for retinol and that the PA has a single binding site for the holoprotein. The combination involves a protein-lipid (RBP-retinol) and a protein-protein (RBP-PA) interaction. Petersen (1971) added the fact that retinyl esters do not bind to RBP.

Goodman (1969) and Petersen (1971) found that under controlled conditions retinol is differentially removed from the RBP-retinol complex at a faster rate, than from the RBP-PA complex. They suggested that no covalent bonding is involved and that the protein-protein interaction serves to stabilize and protect the bound retinol molecule.

1.5) Intra-cellular binding proteins

A number of tissues from rats, human and other species contain a soluble intra-cellular protein with binding specificity for retinol (cellular retinol-binding protein, CRBP), and for a similar protein with binding specificity for retinoic acid (Cellular retinoic acid-binding protein, CRABP) (Goodman, 1984). Both CRBP and CRABP have a molecular weight close to 14,600 and

single binding sites for one molecule of retinoid ligand. The intra-cellular binding proteins differ in a number of major ways from plasma RBP (Goodman, 1984). Ong et al (1982) indicated that CRBP is not tissue specific. Chytil, (1984) found that CRABP allows specific transfer of retinoic acid into the nucleus and to chromatin which was not influenced by CRBP.

Chytil and Ong (1984), Roberts and Sporn (1984) and Lotan (1980) investigated the possibility that there is a relationship between the intracellular retinoid-binding proteins and cancer, or between the proteins and the anti-carcinogenic activity of retinoids. Chytil and Ong (1979) suggested that the intracellular binding proteins may play a direct role in the biological expression of vitamin A activity (eg analogous to steroid hormone receptors).

As indicated later, retinoids affect cell differentiation and proliferation, and are thought to affect gene expression in target cells (Roberts and Sporn, 1984). Kato et al (1985) in a recent report, found that both cellular binding proteins were present in all tissues and organs examined. They suggest that both proteins are involved in critical biochemical functions in many types of cells throughout the body. The nature of these functions, and of the regulatory processes that control the tissue levels of CRBP and CRABP, needs more investigation.

1.6) Biological Action of Vitamin A

Vitamin A (retinol) is necessary for normal vision, reproduction and maintenance of function of the epithelial cells. In general vitamins function in the body as co-factors to enzymes involved in intermediary metabolism.

Figure 4 illustrates the biological action of the three natural retinoids.

Retinal, which is responsible for the visual cycle, is reversibly formed from retinol in the body. The conversion of retinol to retinoic acid is irreversible. In the visual process, vitamin A plays a very special role. Vitamin A, in the form of its aldehyde, retinal, combines with the protein opsin to form rhodopsin (Weber, 1983). Numerous studies in vivo and in vitro have established that vitamin A (retinol and retinoic acid) is required for the normal differentiation and maintenance of epithelial tissues; however, the molecular mechanism of these effects have not yet been revealed (Lotan, 1980). Mueller et al (1978) explained that epithelial tissues undergo continuous replacement, and cells are progressively changing their phenotype, probably in response to hormonal, nutritional or other environmental stimuli. These factors may influence specific metabolic pathways directly or they may modify metabolism by altering the expression of genes, thus producing new proteins and/or glycoconjugates. In several tissues exposure to certain hormones may induce proliferation of specific cells. During cell replication inductive events may lead to irreversible changes in cell phenotypes due to alterations in the spectrum of their inducible genes.

Vitamin A appears to induce proliferation and differentiation of the basal germinative layer of epithelial cells and, according to this, its mechanism of action is similar to the action of steroid hormones.

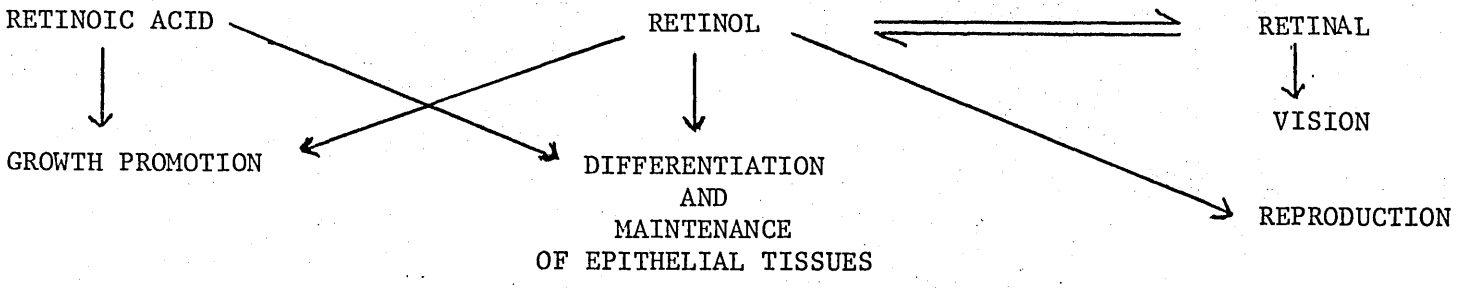


FIGURE 4

The biological action of vitamin A (Mills, 1983)

Mills (1983) studied the effect of vitamin A on cellular differentiation under conditions of deficiency and excess of vitamin A. Deficiency results in squamous metaplasia in the epithelium of the different organs. Excess of vitamin A suppresses keratinisation of epithelial tissue, and also causes a variety of toxic effects (Bollag, 1974). Retinol is accumulated by the liver, and prolonged, chronic ingestion of high doses of vitamin A results in the destruction of liver parenchymal cells. Acute ingestion of high doses may cause brain-stem oedema, leading to headaches, then coma, followed by death (Hicks, 1983).

In an attempt to avoid the toxicity, chemical modification has been tried, usually of the cyclic or polar end groups of retinol. This has resulted in numerous compounds, called retinoids (Rustin, 1983). These synthetic retinoids are not stored in liver, so they do not appear to damage the organ (Rustin, 1983). Retinoids have been used in treatment of skin disease and certain types of cancer, and also in studies of the prevention of carcinogenesis (Meyskens, 1982; Kessler and Meyskens 1984 ; Meyskens et al 1985). Further studies are needed to elucidate their role, fate and their relationships to cell differentiation or proliferation.

1.7) Deficiency of vitamin A

Vitamin A is an essential substance, and for the lack of it many people in the world suffer ill-health, blindness or even death. Vitamin A deficiency seems to be the most common nutritional deficiency disease in the world. The most tragic manifestation of the deficiency is blindness in young children. This occurs since vitamin A operates in vision as retinaldehyde

(Section 1.6), which forms the chromophoric group of visual pigment. If there is insufficient vitamin A, inadequate amounts of rhodopsin visual pigment are formed. In man, rods which are the location of rhodopsin are sensitive to light of low intensity, and this is first affected. This is followed by xerophthalmia or dryness of the eyes, and then by keratomalacia with melting or dense scarring of the cornea (Pirie, 1983).

Vitamin A deficiency leads to damage to epithelial tissues, and apparently also to nerves and bony structures (Aykroyd, 1958). Oomen (1958) hypothesised that many clinical phenomena may be conditioned by vitamin A deficiency eg urinary calculus, bronchopneumonia, and fatty infiltration of the liver. These suggestions have not been confirmed.

Most available evidence suggests that the course of Vitamin A deficiency is due to reduced dietary intake of vitamin itself. Gopalan et al (1960); Venkatachalam and Gopalan (1960) suggested that there are other factors playing a significant role in vitamin A deficiency. Because of the inter-relationship between protein nutritional status and vitamin A metabolism, it has been suggested that protein intake is perhaps one of the factors which may be related to vitamin A deficiency. With protein deficiency, a poor absorption of vitamin A and carotenes from the diet may also occur (Srikantia, 1975).

Infection can play a role in the development of vitamin A deficiency, because infections are known to increase the requirement for many nutrients including vitamin A, and alter their metabolism (Srikantia, 1975).

There are several infectious diseases associated with xerophthalmia. The commonest is upper respiratory disease with fever and cough, and diarrhoea (Pirie, 1983), since vitamin A deficiency causes keratinization of epithelium in the trachea and loss of mucus cells and thinning of the gut epithelium.

Sauter, (1976) found that measles also seems to lead to keratomalacia. Dosseter and Whittle (1975) explained that the measles virus may infect the cornea, and fever will reduce vitamin A transport, since with stimulation protein-energy malnutrition will increase and this will be made worse by the loss of protein through the gut wall.

Sivakumar and Reddy (1972) indicated that absorption of vitamin A is impaired in infectious diseases, and serum vitamin A and proteins are depressed (National Institute of Nutrition, Hyderabad, 1980). Marks (1968) suggested that transport of vitamin A may be affected as a result of depletion of carrier proteins.

Loss of resistance to infection has been established in animals as well as in man in cases of vitamin A deficiency (Clausen, 1935). He believed that an early adequate diet, particularly one rich in vitamin A, tends to prevent the development of vitamin A deficiency and minimise the effect of infection.

Finally, the degree of vitamin A deficiency varies from one area to another and from one animal species to another. The deficiency in the same species varies according to age, sex and environmental condition. To treat vitamin A deficiency, a high

daily intake is needed, as soon as it occurs. However, due to the toxicity of high doses of vitamin A, the treatment must be handled with care.

1.8) Assessment of vitamin A status

For the past 10 years, workers have been developing new or modified methods, with a view to providing information for the evaluation of nutritional status.

Sauberlich et al (1974) indicated that for many nutrients, biochemical laboratory techniques have been investigated, developed and employed in assessing nutritional status. The majority of these techniques involved:-

a) measurement of the nutrient level in the blood

b) measurement of the urinary excretion rate of the nutrient

c) measurement of the urinary metabolites of the nutrient.

d) measurement in blood or urine of abnormal metabolic products resulting from deficient or submarginal intakes of the nutrient

e) measurement of changes in blood components or enzyme activities that can be related to intakes of the nutrient.

f) load, saturation and isotopic tests.

In the past, the assessment of vitamin A status usually involved examining for night blindness or detecting clinical eye-signs of vitamin A deficiency (Section 1.7).

Rodriguez and Irwin,(1972), Peterson et al (1974), Carney and Russell, (1980) used the consequent increase in visual threshold after intake of vitamin A as a measure of vitamin A

status, and vitamin A deficiency has been detected successfully (Russell et al 1973, Carney and Russell, 1980). This could be a valuable measurement when considered with plasma retinol concentration.

Dark adaptation can be faulty for reasons other than vitamin A deficiency (Morrison et al 1978). It would not be practicable to use this test widely because of the cost of the apparatus and co-operation of the subjects who are mostly young children.

Another aid in determining vitamin A status is the assessment of dietary intake. Calculating the dietary intake of vitamin A and provitamin is difficult, because of the different content of carotenoid in food, the maturation effect on carotene content, the rapid conversion of provitamin A to inactive carotenoids biologically, and isomerization and losses during storage and food preparation. The estimation from food composition therefore provides only a qualitative indicator (Olson, 1982). These tests are helpful in confirming vitamin A deficiency but are ineffective in evaluating marginal or normal states of vitamin A nutrition.

Measurement of plasma retinol concentration may help in the determination of vitamin A status. Blood samples can be readily obtained for screening purposes and for sequential studies. Although clinical vitamin A deficiency is always associated with a low plasma retinol, there are limitations in interpreting plasma concentrations. The plasma retinol concentrations can rise and fall for reasons unconnected with the vitamin A status of the body. If dietary protein is inadequate,

RBP biosynthesised in the liver will be affected and plasma retinol concentration will also be affected (Arroyave et al 1961, Glover and Muhilal, 1976). Also retinoids other than retinol, depress the plasma retinol concentration as a consequence of diminishing the demand of tissues for retinol itself (Underwood, 1974, Keilson et al 1979, Loerch et al 1979). Various diseases can affect the output of the retinol-RBP complex by the liver or its metabolism by the kidney; thus lowering or raising the plasma retinol concentration (Smith and Goodman, 1971, Kindler 1972, Underwood, 1974).

A new approach is carried out by Loerch et al (1979) 'relative dose response', based on the increase in the plasma retinol concentration brought about by a dose of vitamin A. If a low plasma retinol concentration is due to lack of reserve in the liver, the relative dose response is high. If little effect occurs, the low level is due to other factors. A more effective approach is the use of isotope dilution assay. When a dose of radioactive retinol is given to rats or humans on a vitamin A-free diet, equilibration between plasma retinol and liver retinol occurs within 7 and 15 to 26 days respectively and total body retinol can be calculated (Rietz et al 1973; Sauberlich et al 1974).

More than 90% of the total body reserve of vitamin A is stored in the liver of a well-nourished individual. Bhat and Lacroix (1983) found that in liver tissue retinol palmitate constituted 79% of the total esters. Thus analysis of liver retinol palmitate concentration should generally provide a good

estimation of total body storage. There are however obvious difficulties in obtaining liver from patients and practical difficulties with autopsy specimens.

It is not known to what extent human liver is capable of concentrating vitamin A. Davies and Moore (1935) gave vitamin A to rats until their livers appeared incapable of holding any more; on examination the levels reached 10,000 IU/g (3,000 µg/g). Smith and Malthus (1962) found that the capacity of human liver to store retinol in the form of retinol esters is very large. They reported values as high as 3,500 µg/g. In general, Pearson (1967) found the ranges are of 100-300 µg/g in well-nourished human populations. Olson (1979) classified the severity of deficient liver retinol levels, as inadequate (less than 20 µg/g), critical (less than 5 µg/g), or absent (less than 0.6 µg/g).

1.9) Daily requirements of vitamin A

Srikantia et al (1975) pointed out that man's requirement of vitamin A was not accurately known. Firstly, there have been few studies designed to evaluate direct human requirements of vitamin A. Secondly, the capacity of human liver to store vitamin A is not known. Finally, although the role of vitamin A in vision has been well established, its role in other physiological functions in the body are not fully understood.

Hume and Krebs (1949) suggested that 750 µg of retinol represents the daily needs of a healthy adult to maintain reasonable body stores in both sexes. In pregnancy there is no need to increase the amount except in lactation, when a minimal

additional amount may be considered necessary. Recommended daily intake HMSO (UK) 1979 was also 750 ug, while recommended daily allowances by the National Academy of Sciences (USA) 1980 was 1000 ug. These amounts are required to maintain an adequate blood concentration and to prevent all deficiency symptoms for adults. An intake above the requirement is considered necessary in order to produce liver storage. Excessive vitamin A is toxic to both children and adults and should be avoided. The committee believes that not more than 2250 ug retinol should be given to adults daily.

1.10) Requirements of vitamin A in disease

Nutrition in general is good for wound healing and other complications occurring after surgery. An adequate supply of energy, amino acids, trace elements, and other micro-nutrients is important. When some of these nutrients are lacking, oedema in the edges of wounds or wound rupture may occur, as an indication of poor healing. Harvey and Howes (1930) observed that surgical wounds in the stomach of the rat healed in relation to the state of nutrition. Bozzetti et al (1975) have demonstrated the improved collagen synthesis in the wounds of patients receiving adequate parenteral nutrition. Tenenbaum et al (1986) found that supplementary vitamin A increases collagen content in the gastrointestinal tract of normal rats, and prevents the decrease in tissue collagen content following injury. Winsey et al (1986) showed that dietary vitamin A supplements prevented the inhibitory effect of preoperative x-irradiation on healing of colon anastomoses. The requirement of vitamin A in disease has

not been thoroughly investigated, but commercial tube feeds for sick persons or those in the post-operative period usually contain the recommended amount for individuals in health.

1.11) Intravenous nutrition (IVN)

In recent years, total parenteral or intravenous nutrition (IVN) has become possible in maintaining nutrition in seriously ill hospital patients. It is widely accepted that a daily supply of energy and nutrients is necessary to maintain a patient in an optimal state of nutrition, and to give a good resistance to illness and trauma (Richards and Kinney, 1977; Kleinberger and Deutsch, 1983). The content of the intravenous solutions should contain the basal nutritional requirements, and also help to make up any losses or deficiencies present.

Intravenous feeding is particularly valuable in situations where oral feeding or tube feeding is not possible. Shenkin and Wretlind (1978) stated that the intravenous nutrients should be in the same quantity and form as are normally transferred to the blood from the intestine after adequate oral feeding.

Dudrick and Rhoads (1971) suggested that IVN may eventually be used to correct metabolic diseases, or to treat patients with malignant disease, by using a diet which starves the tumour, but at the same time supports the patient's nutrition.

Intravenous nutrition consists of the different nutrients, water, amino acid, fats, carbohydrates, minerals and vitamins. For efficient utilisation of all nutrients, they should

be administered at optimal doses to correct existing deficiency, and to prevent the occurrence of new deficiencies during nutritional support.

Vitamin A delivery in intravenous solution has been studied by many workers. Allwood (1982) found that the stability of vitamins in TPN depends on a number of factors, including adsorption to the container, the effects of ultraviolet light and the presence of trace elements. Hartline and Zachman (1976) found that the major factor in decreasing vitamin A administration to the patient was absorption of the vitamin to the chamber and tubing of the administration bottle. This was confirmed by Gillis et al (1983) who suggested a re-examination of the method of solubilization of fat-soluble vitamin and the materials used in the manufacture of clinical infusion equipment. Because of the variations of vitamin A stability, individual workers require to demonstrate its stability in the conditions of study.

1.12) Nutrition and Cancer

The relationship between diet and nutrition and their effect on cancer received little attention before 1942 when Tannenbaum and later Ross and Bras (1965), studied the effect of diet in reducing animal tumours. The relationship between dietary factors and carcinogenesis in humans has been actively studied since then.

Epidemiologists and experimentalists have found that nutrition in general is related to the development of cancer in three ways (Reddy et al 1980):-

i) food additives or contaminants may act as carcinogens or co-carcinogens or both.

ii) nutrient deficiencies may lead to biochemical alteration

iii) change in the intake of selected macro-nutrients may produce metabolic and biochemical abnormalities, which increase risk of cancer.

Rivlin (1982) tried to explain the relationship between nutrition and cancer and suggested that it may be useful to consider the relationship between at least four major items.

First, some dietary factor may influence the development of certain tumours.

Second, the development of cancer often has major effects upon the nutritional status of patients.

Third, the treatment of cancer may effect nutritional status.

Fourth, great advances have been made in the nutritional management of patients with cancer using intravenous, enteral or oral feeding.

Although the relationship between nutrition and cancer is complex, they interact in a number of important ways and to an increasing extent nutritional factors have been found useful in both the prevention and treatment of cancer.

1.13) The relationship between vitamin A and cancer

Vitamin A is required for normal growth and differentiation of epithelial tissues, vision and reproduction, as described in Section 1.6 earlier. Farber, (1981) studied a two step model of carcinogenesis on which most of the understanding of the action of retinoids has evolved (i) initiation: the irreversible genetic alteration of a target cell by carcinogen and (ii) promotion, which results in a phenotypic expression of that genetic alteration. Krinsky and Deneke, (1982) Nettesheim and Williams, (1976) suggest that vitamin A may interact with initiation of a cancer either by direct involvement with the initiator or by increasing susceptibility to carcinogens due to vitamin A deficiency.

The antipromotion effects of retinoids may occur through at least three different mechanisms. Boutwell, (1982) found that retinoic acid (retinoids) inhibited ornithine decarboxylase activity, a key enzyme in the process of tumour promotion. Sporn, (1980), Elias and Williams, (1981) hypothesized that retinoids may directly compete with promoting agents for control of cellular differentiation by effects on cellular protein synthesis. Sporn and Newton (1981) showed that retinoids block the effects of transforming growth factors in vitro, and may compete with growth factor to prevent expression of malignant growth.

Bjelke and his colleagues (1975) found an inverse association between the incidence of lung cancer and the intake of dietary vitamin A. He also found that decreased vitamin A intake was associated with an increased risk of cancer of the

colon (Bjelke, 1974). This negative association may be underestimated because of inaccuracy in measuring the dietary variable. Peto et al (1981) indicated that dietary beta-carotene correlated inversely with human cancer risks, and if dietary beta-carotene is truly protective there are a number of theoretical mechanisms where it might act. Some of these mechanisms do not directly involve its 'provitamin A' activity.

On the other hand measurement of retinol levels in serum may reflect a relationship between vitamin A and cancer. Atukorala et al (1979) in a retrospective study of serum retinol, found low serum concentrations in patients with lung cancer. Ibrahim et al (1977); Wahi et al (1962) found low serum concentration of retinol in patients with oral cancer. In a prospective study, low serum retinol levels were associated with an increased risk of lung cancer (Wald et al 1980). The association was greatest for men who developed lung cancer. Basu et al (1976); Atukorala et al (1979) suggested that the low levels may have been a result of the cancer rather than a precursor.

Kummet and Meyskens (1983) supported the conclusion that vitamin A is a natural inhibitor for the development of cancer, but due to the multiple factors which contribute to the development of cancer, it is difficult to distinguish the exact role that vitamin A plays. As described earlier, due to toxicity, the side effect of using vitamin A as treatment for cancer, synthetic compounds (retinoids) have been developed. Further careful studies are needed in future to establish the use of these compounds in preventing cancer.

1.14) Role of cholesterol in cancer and relation to vitamin A

Dietary cholesterol and vitamin A are both transported from the intestine in chylomicrons produced during absorption of fat (Goodman, 1962, Redgrave, 1970). Both cholesterol and vitamin A are deposited in the liver after catabolism of the chylomicrons (Goodman et al 1965, Fidge et al 1968). The vitamin A and cholesterol are initially removed from the hepatic sinusoid into parenchymal cells. Vitamin A is presumably then transferred from the parenchymal cells into the perisinusoidal fat-storage cells. The fat-storage cells appear to play no part in the metabolism of chylomicron cholesterol, which has been shown to be localised in the non-phagocytic fraction of liver cells (Redgrave and Vakakis, 1976; Redgrave, 1970; Nilsson and Zilversmit, 1971).

In human blood, most cholesterol is found in two types of lipoprotein particles, low density lipoprotein (LDL) and high density lipoprotein (HDL). These two lipoproteins have opposing physiological roles (Peto, 1981).

In recent years, considerable interest has been given to the association between low serum cholesterol concentration and the increased incidence of cancer, especially colon/rectal cancer (Williams et al 1981) and the correlation between serum concentration of cholesterol and the retinoids (Kark et al 1982).

There are several explanations for these associations. Kark et al (1982) suggested that the inverse relationship between serum cholesterol and cancer incidence may be secondary to an association of serum retinol with cancer. This suggested that the inverse relationship may reflect a vitamin A/cancer

relationship by way of the correlation between serum retinol and cholesterol. Smith and Hoggards (1981) supported this, and indicated that the relationship is with LDL-cholesterol. They found also a possible link between serum beta-carotene and serum cholesterol. Adams et al (1985) indicated that association between low serum cholesterol concentration and cancer risk is likely to be indirect, and may be mediated through vitamin A metabolism. One possible mediator is carotene, since LDL is involved in the transport of carotene. Most evidence of a relationship between cholesterol concentration and cancer has implicated primarily total cholesterol and LDL-cholesterol and not HDL-cholesterol, (Wald et al 1980; Rose and Shipley, 1980, consistent with the suggestion that low cholesterol level may be a metabolic consequence of cancer rather than a precursor.

Rose et al (1974) in a six-country prospective study identified only 90 persons with coronary heart disease who also in due course developed colon cancer. Cruse et al (1979) suggested that cholesterol may act as a co-carcinogen within the colon. Unfortunately, many details remain obscure. Further studies on dietary intake of fat, coronary heart disease, beta-carotene and retinol metabolism and the relation to cancer are needed.

1.15) The objects of the present study were:-

1) To develop a specific high pressure liquid chromatography method to separate retinol and its metabolites.

2) To investigate the effect of nutritional status, on serum and liver vitamin A.

3) To investigate the role of vitamin A status in cancer, using serum and liver vitamin A measurements.

2) Methods

Statistical Methods

In general, results throughout this thesis are expressed as mean ± SD (standard deviation). Comparison of means has been made by Student's t-test. Where results were not normally distributed, median and range is stated and comparison of groups has been made by Mann-Whitney test.

Analytical Methods

2.1) Methods of measuring Retinol

2.1.1) Colorimetric Method

The Carr-Price method (1926) has been extensively used and involves the treatment of vitamin A with antimony trichloride. The blue colour thus obtained has a maximum absorbance at 620 mμ.

This method has been widely used in the past but there are disadvantages. The colour formed is temporary and must be read within 5-15 seconds. The method is extremely sensitive to moisture, which results in a turbid solution (Moore, 1957). It is not specific for retinol, since carotenes are measured at the same time and a correction is made for the colour given. The reagent is corrosive, is a source of danger to the instrument and difficulty may arise in the cleaning of cuvettes. The method is therefore unsuitable for routine use.

Because of these limitations, a variety of methods for the determination of vitamin A in serum and food have been applied and modifications to the Carr-Price method have been made.

Bessey et al (1946) developed an UV spectrophotometric method to determine vitamin A and carotene by reading the extinction at 325 nm and 460 nm respectively before and after irradiation of the serum with ultraviolet light of a wavelength between 310 and 400 nm. Vitamin A and carotene were destroyed under these conditions. These determinations required expensive

equipment and haemolysed blood has been found to give higher carotene values. Results tend to be unreliable, due to the higher blanks after irradiation (Sobel and Snow, 1947).

The Carr-Price method and its modification are unsuitable for measuring vitamin A in food products, because of problems with turbidity and non-specific interference.

Needl-Pearson (1963) developed a method where trifluoroacetic acid (TFA) reacts with vitamin A, to form a compound with a blue colour. This blue colour has a maximum absorption at 616 mu. The procedure is the same as the Carr-Price method except that TFA replaces the $SbCl_3$. This method shows increased sensitivity and specificity, but since carotene reacts with TFA a correction is applied. A large percentage of stored specimens contain an artefact which gives a colour with TFA, similar to that given by vitamin A.

Most of these experiments measured total vitamin A and a correction is needed to obtain the true retinol value, because of the colour given by carotene. The colour produced is unstable. Precision and accuracy are poor due to interference.

2.1.2) Fluorimetric method

Many procedures have been published using the property that vitamin A (Retinol) and its derivatives (ester forms) fluoresce in ultraviolet light.

Thompson et al (1971) developed a method using excitation wavelengths of 330 and and 360 nm and emission was measured at 480 nm. They tested the interference of other derivatives, and only phytofluene (a carotene-like pigment present in tomatoes) had a large value of fluorescence, which

might interfere with the retinol results. The comparison of their method with other previous workers gave a good correlation except that due to phytofluene interference a correction formula was needed. They were unable to assess accuracy as the true level of retinol in the blood was not known.

Drujan et al (1968) obtained normal values for vitamin A (Retinol) in human blood samples. They suggested that there was probably no interference due to phytofluene. The diet of the volunteers was very low in vitamin A in this study.

Kahan (1966) used an extraction system of water:ethanol-:cyclohexane (1:5:5). He found that there was a linear relationship between fluorescence and concentration of retinol acetate within the range of 0.2-300 µmol/l. The deviation at levels higher than 300 µmol/l is due to concentration quenching. He claimed to have demonstrated specificity by confirming that no interference occurred from haemoglobin, bilirubin, cholesterol, other fat-soluble vitamins and carotenoids, which had no detectable fluorescence. Hansen and Warwick (1966 and 1968) confirmed that finding, although the retinol level was above the accepted normal value, due to the presence of phytofluene.

Leitner et al (1960) estimated vitamin A and carotenoids. They found that sex, food as well as season affected the levels of vitamin A and carotenoids in blood samples.

Previous workers have developed their fluorimetric methods using different solvents, excitation and emission wavelengths. They did not give adequate accuracy due to the presence of phytofluene, also there was no mention of precision or percentage recovery and they used a formula to correct interference

of phytofluene. The treatment of the sample and standard may have been different, since standards were extracted from aqueous solutions rather than from preparations using vitamin free serum.

As part of the present study, a comparison was performed between a fluorescence method and a high performance liquid chromatography method (HPLC).

2.1.2.1 Fluorescence method for vitamin A analysis, used in this study

Absolute alcohol "Analar" cyclohexane (fluorescence grade), BDH, England, retinol acetate (Sigma). Perkin Elmer MPF 3 spectrofluorimeter used with setting for retinol excitation 340 nm, emission 470 nm with slit width 10 nm for both , and sensitivity setting as required.

Method

The method used for measuring vitamin A in plasma was a modification of that of Kahan (1966) and Thompson et al (1973). The principle is that the plasma sample is diluted in aqueous ethanol and retinol extracted into cyclohexane and determined by fluorimetry.

To 1 ml of distilled water, add 500 ul of distilled water, plasma or working standard of retinol acetate.

5 ml of ethanol 99.5% is added, the solution mixed by shaking, then 4 ml of cyclohexane is added, the mixture shaken well, centrifuged and the upper cyclohexane layer removed. The fluorescence was measured with a setting of zero with blank solution and 50 with standard.

$$\mu\text{mol/l retinol} = \frac{\text{Fluorescence intensity of sample}}{\text{Fluorescence intensity of standard}} \times C$$

Where C = concentration of standard

2.1.2.2 Vitamin-free serum

The method used was adapted from Schade, (1981). 15 grams activated charcoal powder (Norit Ol, Searle) was added to 100 ml serum pool, and stirred with a magnetic rod overnight at 4°C. The preparation was ultracentrifuged at 40,000 rev/min at 10°C for half an hour. The supernatant was cleared with crystalline cellulose and centrifuged (sometimes serum was so clear after ultracentrifugation that this step was not required). The mixture was checked on HPLC for absence of retinol, then 0.1% sodium azide (NaN_3) was added and aliquots were stored at -20°C. Figures 5-7 show serum before and after charcoal, compared with vitamin-free sera from horse serum (Bovine, Wellcome), from Victoria Infirmary, Glasgow*, from Western Infirmary, Glasgow+ (dried plasma) and from serum exposed to UV light for one hour, respectively.

Results

Different types of charcoal were used (10-18 mesh) activated for gas absorption (BDH). The best results obtained by us were with activated charcoal powder (Norit Ol, Hopkins, Williams). Different types of filter were used also, as suggested by Schade (1981), but ultracentrifugation was most effective at removing charcoal. Figure 5 shows that a vitamin-free serum was prepared by this method.

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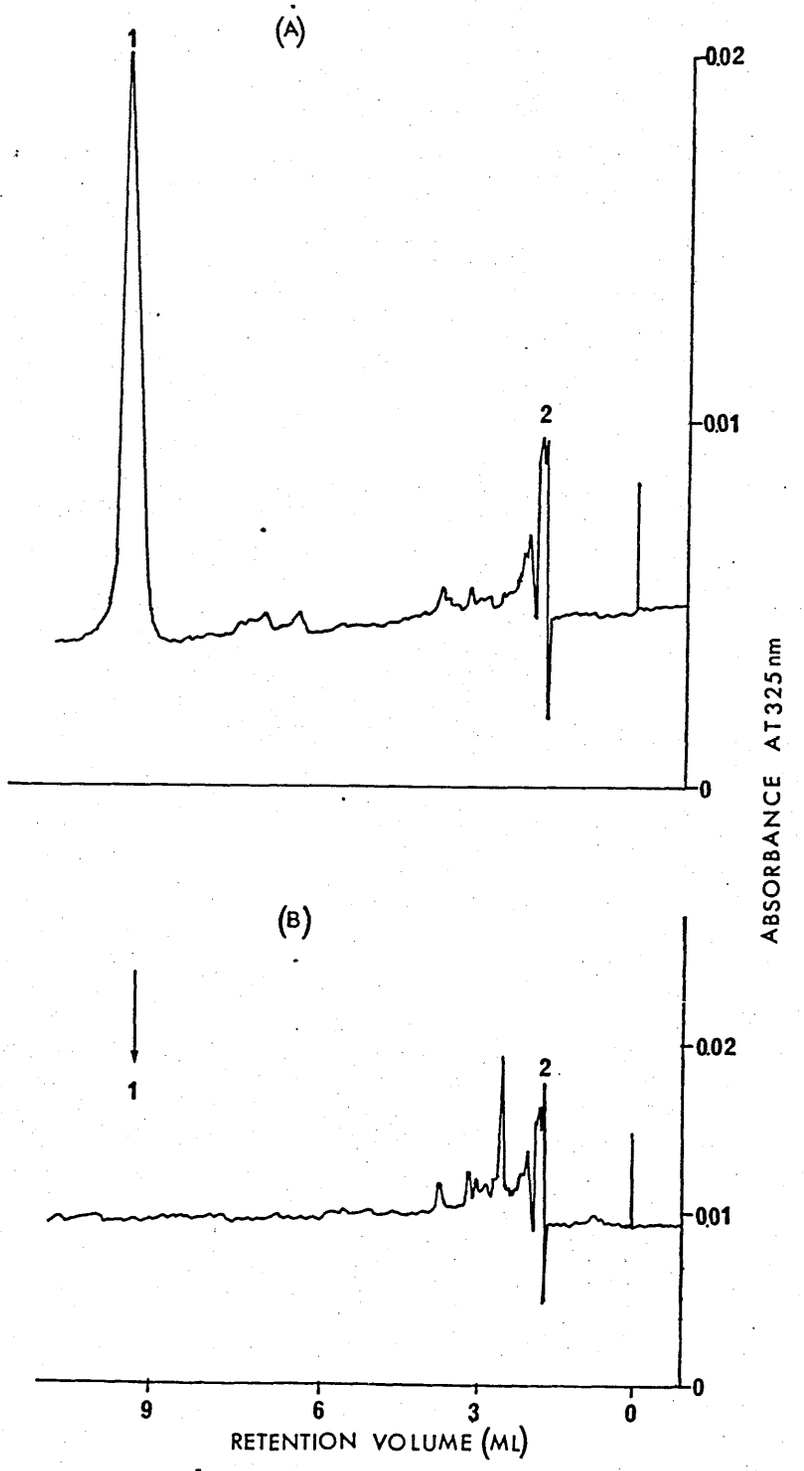


Figure 5: Chromatograph of human serum retinol before (A) and after (B) charcoal treatment

Peak identification:-

1 - Retinol
2 - solvent peak

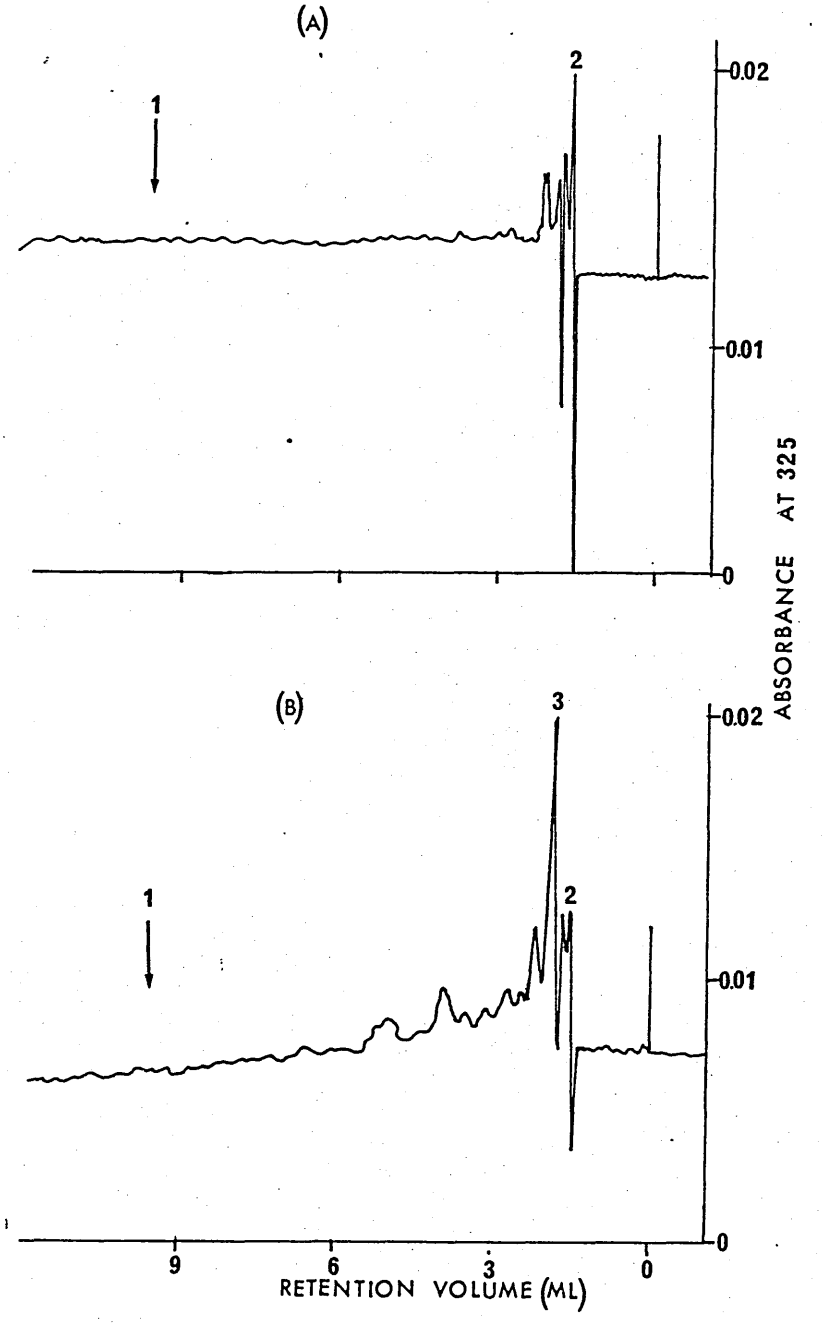


Figure 6: Chromatograph of vitamin-free serum
(A) charcoal treated bovinehorse serum
(B) prepared by Victoria Infirmary, Glasgow

Peak identification

1 - position of retinol peak
2 - solvent peak
3 - unidentified peak

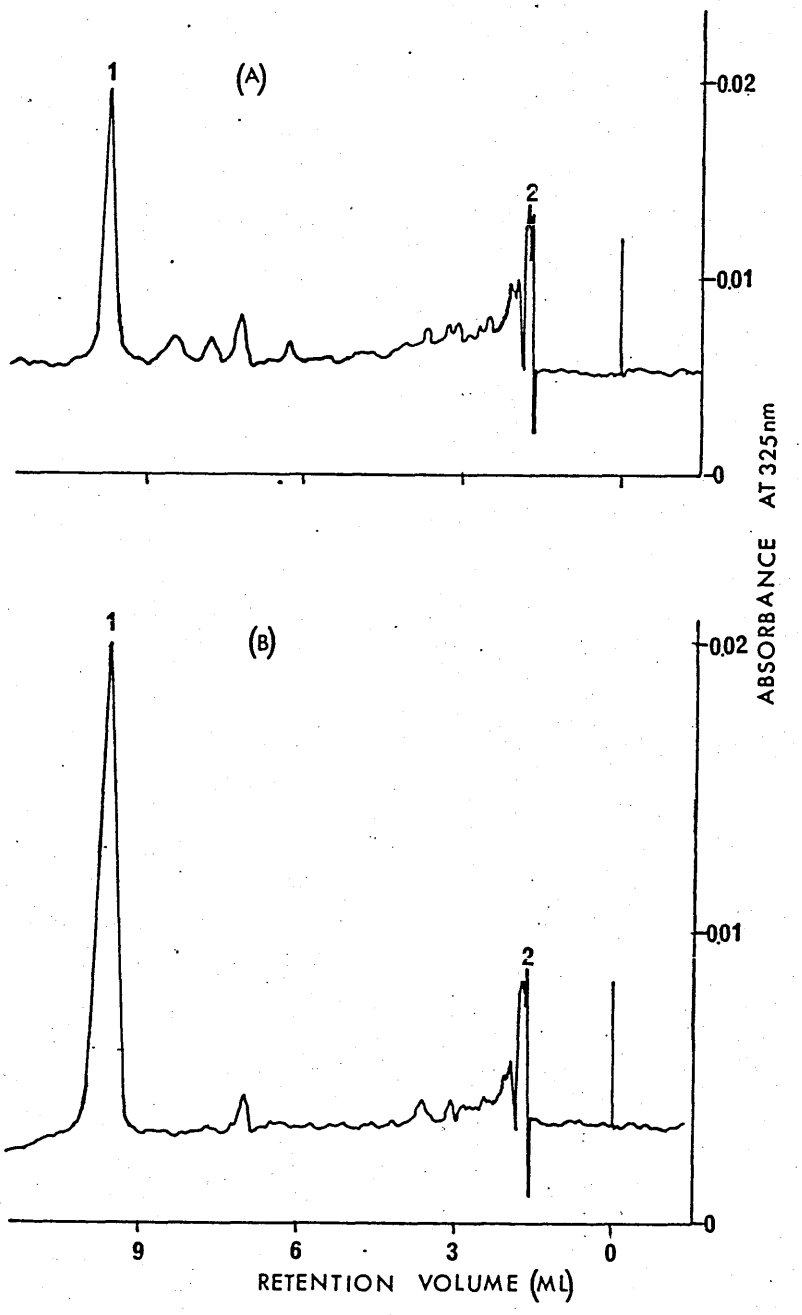


Figure 7: Chromatograph of vitamin-free serum
(A) of Western Infirmary, Glasgow,
(B) serum treated for one hour under UV light

Peak identification:-

1 - Retinol
2 - solvent peak

2.1.2.3 Fluorescence Assay:

1) linearity and accuracy of method

A calibration curve was prepared using retinol acetate in alcohol and was found to be linear (Figure 8). A calibration curve was also prepared for retinol acetate using vitamin-free serum. It was also linear, except that there was a reduction compared with retinol acetate standard in alcohol. A retinol standard at 10 µmol/l in vitamin free serum gave a value of 3.6 µmol/l against the retinol acetate standard in alcohol and a reading of 6.7 µmol/l against the retinol acetate standard in serum. This suggests that the standards have been more poorly extracted from serum, and that retinol and retinol acetate may be extracted to different extents.

2) Comparison of retinol with retinol acetate as standard

Many workers have used retinol acetate as standard, although the retinoid in serum is retinol. Figure 9 and Table 1 shows the actual retinol concentration compared with the measured concentration by fluorescence against retinol acetate standard.

The table and figure show that increasing concentrations of retinol, lead to linear increases in fluorescence. However, the calculated concentration of retinol is very low, relative to its actual concentration. A retinol standard was therefore investigated.

3) Retinol standard curve (linearity)

A calibration curve was prepared using retinol in alcohol as standard (Figure 10). It was found linear over a wide range (0-10.5 µmol/l). This was compared with that of standard

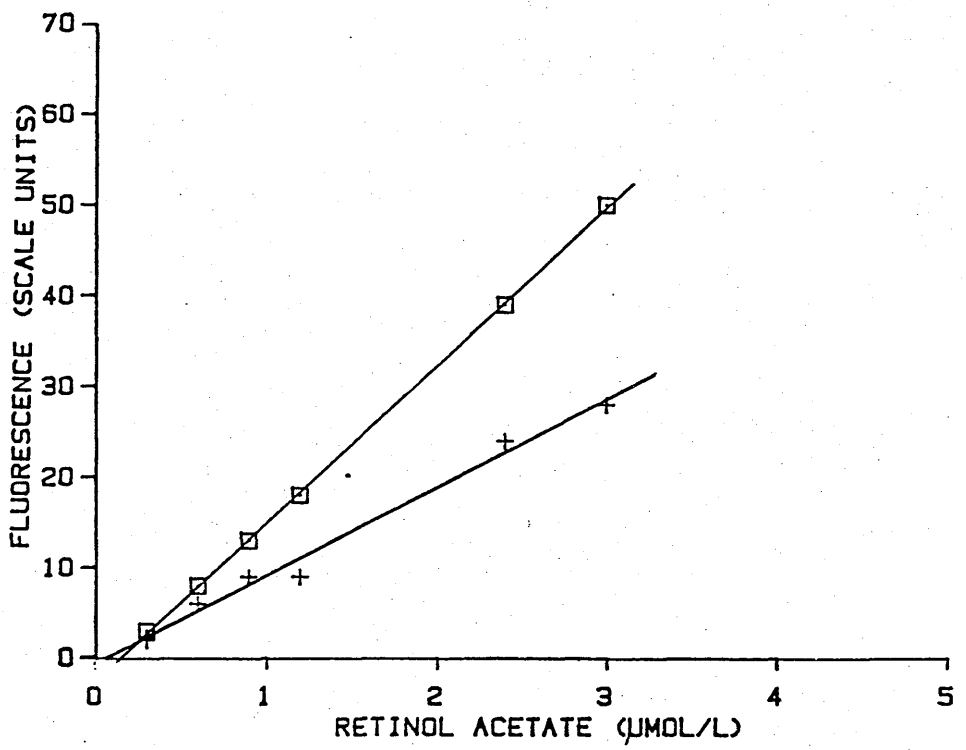


Figure 8 : Fluorometric assay of retinol acetate in alcohol (□) and vitamin-free serum (+)

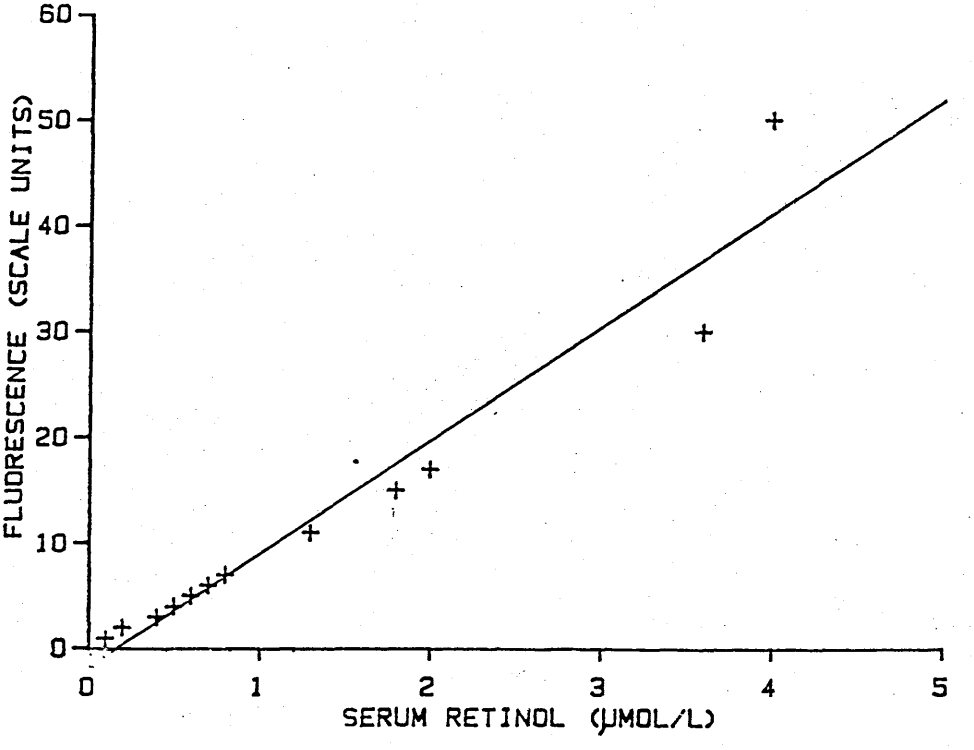


Figure 9: Fluorometric assay of serum retinol concentration - A calibration curve using retinol acetate standard in alcohol.

TABLE 1

MEASURING DIFFERENT RETINOL CONCENTRATIONS AGAINST RETINOL ACETATE STANDARD

Actual Concentration of retinol std (μmol/l)	Fluorescence (units)	Calculated retinol conc^{n} (μmol/l)
0.4	1	0.1
0.7	2	0.2
1.0	3	0.4
1.1	4	0.5
1.4	5	0.6
1.8	6	0.7
2.1	7	0.8
3.2	11	1.3
3.5	11	1.3
4.2	15	1.8
5.3	17	2.0
10.5	30	3.6
Retinol acetate (4.0)	50	-

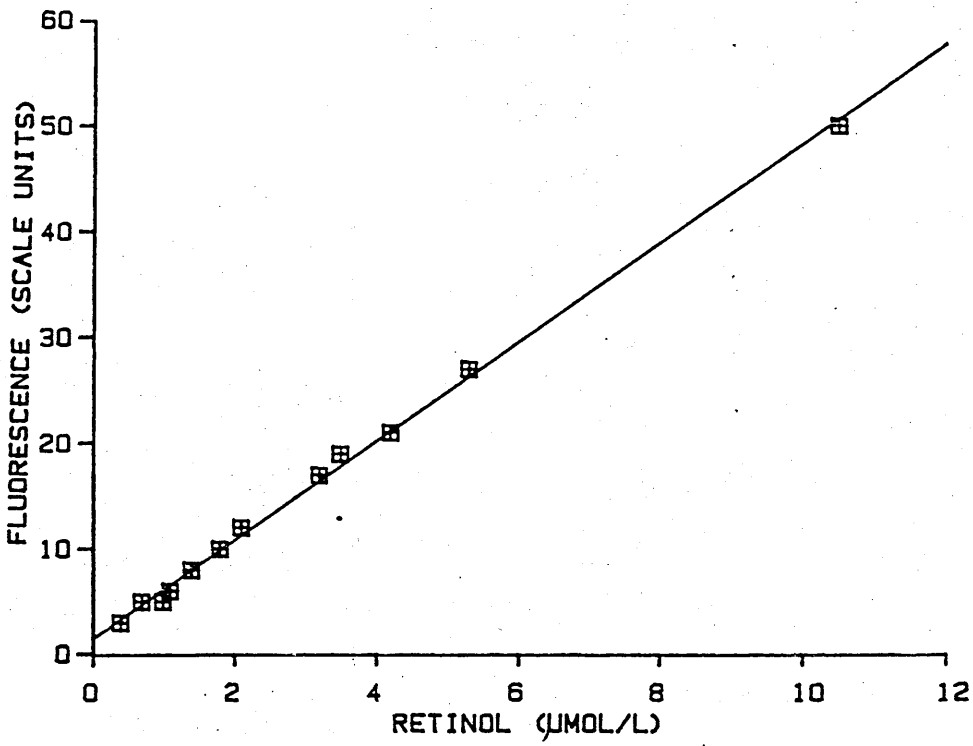


Figure 10: Fluorometric assay of retinol. A calibration curve of standard retinol in alcohol (□), and in serum (+)

retinol in vitamin-free serum. No difference in fluorescence was obtained (Table 2). This contrasts with the reduction in retinol acetate extraction from vitamin-free serum.

Previous studies have suggested that either hexane or cyclohexane may be used for extraction (Kishi et al 1981). The fluorescence of retinol standard extracted with these solvents was studied (Table 3).

It is clear that fluorescence is much greater when hexane is used as solvent. Other retinol derivatives were therefore studied in a similar way (Table 4). Retinol and carotene are more efficiently extracted by hexane, whereas retinal appears to be equally extracted by cyclohexane and hexane. Retinoic acid does not appear to fluoresce under these conditions.

Discussion

These studies have indicated the importance of using retinol as standard rather than the commonly used retinol acetate. If we compared the fluorescence of the same concentration of retinol in Tables 1 and 2, which were read against retinol acetate standard and retinol standard respectively, it is found that the retinol form of the vitamin gives a fluorimetric response about 1.7 times that of the retinol acetate form. This result agrees with that of Garry et al (1970) who suggested the use of retinol as the primary standard in the fluorimetric procedure, rather than the more stable acetate derivatives. Kishi et al (1981) used hexane instead of cyclohexane for extracting retinol from plasma, whereas retinol acetate was used as standard. In this study we found that the fluorescence increased

TABLE 2

STANDARD CURVE OF RETINOL

Actual Concentration of retinol std (μmol/l)	Fluorescence in alcohol (scale units)	Fluorescence in vitamin-free serum (scale units)
0.4	3	3
0.7	5	5
1.0	5	5
1.1	6	6
1.4	8	8
1.8	10	10
2.1	12	12
3.2	17	17
3.5	19	19
4.2	21	21
5.3	27	27
10.5	50	50

TABLE 3

FLUORESCENCE OF RETINOL IN CYCLOHEXANE AND HEXANE

Actual concentration of retinol std (μmol/l)	Fluorescence in cyclohexane (scale units)	Fluorescence in Hexane (scale units)
0.4	1	4
1.0	3	10
2.1	7	20
4.2	15	40
10.5	30	110
Retinol acetate	50	50

TABLE 4

COMPARISON OF RETINOL, RETINAL, RETINOIC ACID, CAROTENE FLUORESCENCE

Actual Concentration (μmol/l)	Fluorescence in cyclohexane (scale units)	Fluorescence in Hexane (scale units)
Retinol (10.5)	30	110
Retinal (10.5)	20	20
Retinoic acid (10.5)	1	0
Carotene (1.1)	11	27
Retinol acetate (4.2)	50	50

3 times with hexane extraction compared to cyclohexane. Hexane therefore extracts more retinol than cyclohexane. In addition, the retinoic acid, retinal and carotene fluoresce less than retinol. Therefore the concentration calculated is less than that added. Frolik and Olson (1984) suggested that retinol and its esters are the only naturally-occurring retinoids that fluoresce under normal conditions.

In this study (Section 3.2.2) we found that triglyceride may effect the fluorimetric method possibly as a quenching factor. This may be due to the large particle-size of triglyceride which scattered the beam causing quenching. The disadvantages of the fluorimetric method were therefore:-

1) the fluorimetric method did not give an accurate value when retinol acetate was used as standard and extraction was less efficient than retinol

2) quenching may occur with some compounds, eg triglyceride and beta carotene and also with haemolysis.

3) a correction formula is used to allow for phytofluene presence.

4) Basu et al (1982) reported that vitamin A, phytofluene or both could be reduced in cancer and the effects could be variable. The results in cancer patients should be confirmed with valid methods of analysis eg HPLC.

5) Garry et al (1970) suggested that phytofluene concentration varied from 0-5 times that of vitamin A.

Because of the disadvantages of the fluorimetric method, we developed an HPLC method. This should be more specific, accurate and precise and could separate all retinol derivatives on one system.

2.1.3) High performance liquid chromatography (HPLC)

2.1.3.1 Background

High performance liquid chromatography (HPLC) has been used for the analysis of retinol and its derivatives. McCormick et al (1978) reported a reverse-phase HPLC procedure and DeRuyter and Deleenheer (1978) used ultraviolet detection at 330 nm with a reverse-phase packed column. The authors extracted retinol and retinyl esters according to the Bligh and Dyer (1959) procedure. The sensitivity of this method was 0.2 µmol/l for retinol and 0.4 µmol/l, for retinyl esters. The authors did not discuss other interferences although they suggested that this method could be used for the assessment of vitamin A absorption and for the determination of serum retinol (normal, subnormal and above normal concentration).

Catignani and Bieri (1983) reported an assay for retinol and alpha-tocopherol in serum or plasma by HPLC in which reverse phase uBondapak C_{18}, 10 um column was used with 95:5 methanol: water as mobile phase. The peak height of vitamins were measured against an internal standard to obtain the quantity of each. According to DeLeenheer et al (1979) 95% of vitamin A in serum is retinol. Esterified forms of vitamin A such as retinol palmitate appear in the blood only after ingestion of a vitamin A-containing meal and can be measured by the method of Catignani

and Bieri (1983). Long retention times are involved and the normal concentrations of the esters was found to be below detection in the post-absorptive state. The authors did not discuss using vitamin-free serum for calibration of the assay but they achieved good recovery when they correlated results with Neeld and Pearson (1963) colourimetric methods using trifluoro-acetic acid. ($r = 0.8$).

Palmskog (1980) reported an assay for determination of two aromatic retinoic acid analogues with anti-psoriatic activity in which a reverse-phase column was used. The sensitivity of this method was 0.035 µmol/l and experimental error below 9%, in a concentration range 0.1-1.6 µmol/l. He believed that a method is needed for the separation of retinol and its derivatives, particularly if the retinoid is used as an anti-cancer drug, to see if there are any other metabolites present in serum. This may also be helpful in analysing toxic effects.

Roberts et al (1978) reported an HPLC assay with excellent recoveries and high resolution, using reverse-phase with a solvent mixture of acetonitrile:water. The quoted sensitivity was 1 nmol/g tissue for retinyl esters and less than 0.3 nmol/g tissue for retinol, using dual-wavelength detection at 365 nm and 325 nm. They achieved a separation of most retinol derivatives in tissue. The problem was the appearance of an artifact peak caused by a refractive index change during the change of mobile phase concentration from 80% to 98% (acetonitrile: water). A further drawback of the HPLC procedure was reported by Frolik et al (1978), who found that the column inlet blocked after 40-50 sample injections.

De Ruyter and De Leenheer (1976) reported an HPLC straight-phase assay to determine retinol in serum using 10 µm (15 cm x 0.2 cm id) column with petroleum ether:dichloromethane:-isopropanol, (80:19.3:0.7) as mobile phase. The quoted sensitivity was 0.2 µmol/l (50 µg/l) and linearity was demonstrated up to 5.2 µmol/l (1.5 mg/l). They achieved a separation of retinol from all other derivatives except retinoic acid, which had k' = 5.2 while retinol k' = 5.0 (Section 2.1.3.2 for k' measurement). They also succeeded in producing vitamin-free serum by irradiating an aliquot of serum sample with long wave UV light (>320 nm) for 3 hours.

Although they produced vitamin-free serum the procedure was standardised by using different values of retinol in alcohol and the analytical recovery of retinol from serum was determined by adding known quantities of retinol to serum.

McCormick et al (1978) achieved a separation of the mixture of four synthetic vitamin compounds - retinoic acid, retinol, retinal and retinol palmitate using reverse-phase µC_{18}-Bondapak column (4 mm id x 30 cm) at 313 nm with 0.01 M sodium acetate methanol: water (80:20) with 90-95% recovery. The procedure took 26 min. This method has been difficult to reproduce in our laboratory (Table 9).

Few of these assays incorporated an internal standard and none of the authors have reported the investigation of possible interference by other drugs. This is surprising in view of the common use of drugs in most patients.

HPLC methods used are shown in Table 5. The drawback of each method can be seen in terms of variable or uncertain recovery, failure to separate retinol metabolites and lack of specificity data.

One of the aims of this study was therefore to develop and validate a sensitive, accurate, precise method for retinol and its derivatives in biological materials.

2.1.3.2 Development of HPLC method for retinol and its derivatives

Materials and Equipment

Materials

Retinol, retinal, retinoic acid, retinol palmitate, carotene and also retinol acetate, were obtained from Sigma Chemical Company (Dorset, England). Hexane, HPLC grade was obtained from Rathburn Chemicals (Walkerburn, Peebleshire, Scotland). Tetrahydrofuran stabilizer with 0.1% quinole, BHA (butylhydroxide anisole) were obtained from Sigma. Methanol and all other reagents were obtained from British Drug Houses (Poole, Dorset, England).

Evaluation of Performance

Definitions: K': the capacity factor or distribution ratio is a function based on retention times, a measure of the capacity of the column to retain a given sample.

$$k' = \frac{t_r - t_o}{t_o} = \frac{t'_R}{t_o}$$

t_o = retention time of the unretained peak(solvent peak)

t_r = retention time of the given peak

t_R = the adjusted retention time

TABLE 5 THE CHARACTERISTICS OF PUBLISHED HPLC METHODS FOR RETINOL AND ITS DERIVATIVES

Author	Type of Column	Separation Achieved	Recovery	Precision	Sensitivity	Linearity	Internal Standard	Limitations	Interference
McCormick et al (1978)	Reverse phase	Retinol, Retinoic acid, Retinal Retinol palmitate	90-95%	-	1 ng of all trans-retinoic acid	-	-	-	not known
Zile and DeLuca (1968)	Straight phase	Retinol, Retinal, Retinoic acid, Retinol acetate	90-100% 60-75% retinal 10-15% retinol acetate and retinol palmitate	-	-	50-500 ng		Retinyl esters	not known
DeRuyta and Deleenheer (1976)	Straight phase	Retinol, Internal standard	91-100%	2.5%	50 µg/l	1.5 mg/l	All trans-9-(4-methoxy-2,3,6, trimethyl-phenyl)-3,7-dimethyl-2,4,6,8-tetraenol).	Retinoic acid interferes with retinol	not known
Bieri et al (1979)	Reverse phase	Retinol, Retinol acetate Retinol palmitate	103.4±5.2%	4.1%	-	-	retinol acetate	Retinoic acid not studied	not known
Western Infirmary (Glasgow)	Reverse phase	Retinol, Retinol acetate	Good recovery	-	-	0-1500 ng/ml =0-5.2 µmol/l	retinol acetate	-	not known
Schade (1981)	Reverse phase	Retinol, retinol acetate	Good recovery	-	-	-	retinol acetate	-	not known

R_S = the resolution of two chromatographic peaks is a measure of their separation.

$$R_S = 2 \frac{t_2 - t_1}{W_1 + W_2}$$

t_1 and t_2 are the retention distances of peaks 1 and 2 respectively. W_1 and W_2 are the peak base widths (in same units as t_1 and t_2) for peaks 1 and 2 respectively (Pryde and Gilbert, 1979).

Choice of retinol acetate as internal standard (IS)

Relevant factors in the choice of retinol acetate as internal standard are that it has a similar structure to the analyte; it is stable; it has an acceptable k' value (Table 12); it is easily extracted; from aqueous solution; it is available commercially; and it is resolved from retinol and its derivatives on the HPLC column (Figure 11). The main advantages of using an IS are that it eliminates errors from pipetting during the evaporation of solvent and the variation in extraction.

Apparatus

Four HPLC systems were used:-

1) Varian HPLC system (Varian Associates, Walton, Thames, England)

2) A Pye-Unicam HPLC system (Cambridge, England) consisting of an LC-XPS single piston reciprocating pump, Alc-UV variable wavelength detector and a PM 8521 single pen recorder.

3) A Gilson HPLC system (Middleton, USA) consisting of a Gilson 302/5S piston pump, Holochrome, HM/HPLC and Rikadenki single pen recorder.

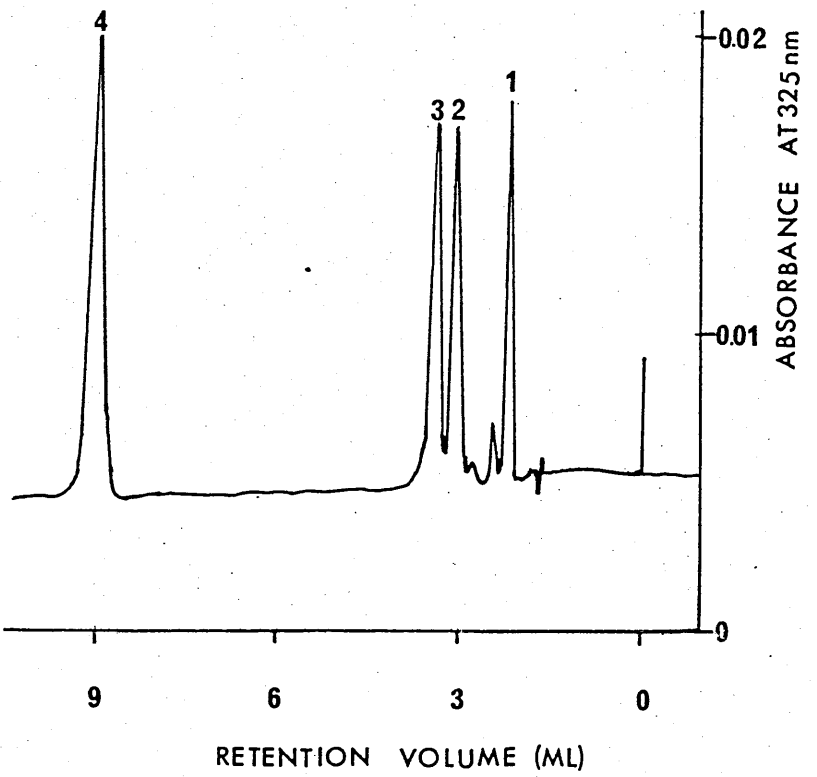


Figure 11:- Chromatography of a mixture of retinol and its metabolites. Peak identities are

1 - retinol acetate (IS)
2 - retinal
3 - retinoic acid
4 - retinol.

4) Cecil instrument C_E 212 reference variable wavelength RE54, servoscribe 15 recorder and Magnus pump.

The stainless steel column (4 mm ID x 25 cm) was obtained from Shandon Southern (Runcorn, England) slurry packed with 5 µ ODS Hypersil (Shandon Southern) using a Shandon slurry packer and fitted with a valve adaptor (Shandon Southern). Also a 3 µ ODS column with same description was used.

Another stainless steel column (4 mm ID x 25 cm) was obtained from Shandon Southern (Runcorn, England), slurry packed with 5 µ silica (Hypersil C002 3H) Shandon Southern. The injection value was fitted with a 20 µl loop.

Methods

Reverse phase HPLC studies

A reverse-phase material (ODS Hypersil) was used to attempt to separate retinol from retinal, retinoic acid, retinol palmitate, and retinol acetate as IS. The liquid chromatographic method was developed as follows:-

a) Polarity investigation

Methanol: water were first used as a mobile phase, to elute the retinol and its derivatives. Increase in polarity was effected by increasing water relative to the methanol. The changes in k' of retinol and its derivatives were studied (Table 6). This study failed to separate retinol from its derivatives, particularly retinal.

b) pH control

The above aqueous solutions were adjusted to pH 5.2 using 0.05 M phosphoric acid, but failed to change k' for retinol and its derivatives.

TABLE 6

COMPARISON OF k', WITH SOLVENTS CONTAINING DIFFERENT PROPORTIONS OF MEOH:WATER

Solvent \ k'	Retinol	Retinoic Acid	Retinal	Retinol palmitate
95:5	2.0	1.0	2.0 & 3.5	*
92:8+	1.75	1.6	1.58	*
90:10	3.0	1.0	3.5	*
85:15	8.0	9.5	8.3	*
80:20	16.8	10.5	16.5	44.0
75:25	29.8	11.5	29.5	*

+ Cecil Instrument used
* Retinol palmitate (Nil) no peak

c) Ion-pair

1-octane sulphonic acid was used as ion-pair at a concentration of 27 mg/l in methanol: water, also 0.01% tetra-n-butylamine, 0.01% DL-N-octdylamine, while 0.1% hexane sulphonic acid, 0.01% tetraethylamino bromide (TEABr), 0.01% Do-decyltrimethylaminobromide (DDTMABr) were used with 0.05 M phosphoric acid (Table 7).

d) Column particle size

3 μ particle size ODS column with same conditions of methanol: water containing 0.05 M phosphoric acid + 0.01% (TEA Br) or 0.01% (DDTMABr) and methanol: water containing 0.01 M sodium acetate were also studied (Table 8)

e) Method of McCormick et al (1978)

We attempted the separation method as described on Page 33 and results are shown in Table 9.

By using different column size particles, pH and different ion-pair as well as the method of McCormick et al (1978) we failed to obtain a different k' for retinol and retinal. Although retention time is changed, there is still an overlap between the other derivatives and retinol.

f) The mixture of methanol: water (85:15) combination was further investigated by adding different ion-pairs to this eluant and k' was studied (Table 10). No improvement in separation was found.

TABLE 7

COMPARISON OF K', BETWEEN SOLVENTS CONTAINING DIFFERENT PROPORTIONS OF METHANOL:WATER WITH ION-PAIR AGENTS USING 5 μm ODS COLUMN

K' / Methanol: water	Retinol	Retinoic Acid	Retinal	Retinol Acetate
95:5	1.0 (1.0)	1.0 (1.0)	1.1 (1.0)	1.5 (1.3)
90:10	2.3 (2.0)	2.3 (2.0)	2.5 (2.1)	3.7 (3.2)
85:15	4.0 (4.1)	4.1 (3.7)	4.2 (4.2)	7.2 (7.4)
80:20	9.3 (9.1)	10.1 (7.3)	9.9 (9.3)	20 (18.5)
75:25	19 (19)	21.2 (11.0)	19.8 (19.3)	took more than 1 hour
70:30	70	high pressure	high pressure	

Solvent A Methanol: water containing 0.05 M phosphoric acid + 0.01% TEA Br ( ) results obtained with solvent B Methanol:water containing 0.05 M phosphoric acid + 0.01% DDTMA Br.

TABLE 8

COMPARISON OF K', BETWEEN SOLVENTS CONTAINING DIFFERENT PROPORTIONS OF METHANOL:WATER WITH ION-PAIR AGENTS USING 3 μm ODS COLUMN

K' / Solvent A or B	Retinol	Retinoic acid	Retinal	Retinol acetate
95:5	1.6 (1.5)	1.9 (0.8)	2.0 (1.5)	30 (3.5)
90:10	3.8 (3.7)	4.5 (1.2)	4.4 (3.8)	8.3 (7.5)
85:15	9.8 (9.0)	11.0 (4.1)	9.9 (9.0)	took time
80:20	21.1 (15.0)	26.2 (11.6)	21.2 (14.7)	
75:25		high pressure		

Solvents were as in Table 7 .

TABLE 9

COMPARISON OF K', USING THE SAME CONDITIONS AS McCormick *et al* 1978

Solvent	Retinol	Retinoic Acid	Retinal
80:20	22.0	5.0	21.0
90:10	3.9	-	3.9

Solvent: 0.1 M sodium acetate methanol:water

TABLE 10

COMPARISON OF K' WITH METHANOL:WATER PROPORTION (85:15) AND DIFFERENT ION PAIR AGENTS.

Compound \ Solvents	A (K')	B (K')	C (K')	D (K')
Retinol	3.8	3.4	3.0	3.2
Retinoic Acid	4.1	3.0	1.9	0.2
Retinal	3.9	3.2	3.0	3.2
Retinol acetate	7.1	5.9	5.4	5.8
Retinol palmitate	After 1 hour and half (nil)			

Solvent A: Methanol:water (85:15) containing 27 mg/l 1-octane sulphonic acid

Solvent B: Methanol:water (85:15) containing 0.01% tetra-N-butylamine

Solvent C: Methanol:water (85:15) containing 0.01% Dodecyltrimethylamine

Solvnet D Methanol:water (85:15) containing 0.01% D-1-N-octdylamine

Straight Phase chromatography

a) TLC: Thin layer chromatography

Thin layer chromatography provides a rapid method of studying mobile phase in a straight-phase system. Dc-plastik-folien (Kieselgel 60 F_{254}) (E. Merck, Dermstadt) was used. 20 µl samples were applied about 0.8 cm from the lower edge of the plate. The plates were developed in a darkened rectangular chamber in 250 ml of solvent by ascending chromatography at room temperature for about 30 min. Immediately upon removal from the chamber, the compounds were detected by exposure to a UV lamp at 254 nm. The R_F of retinol and its derivatives were studied with different solvents (Table 11).

Definition

The R_f value is the ratio of the distance travelled by the solute to the distance travelled by the solvent front:

$$Rf = \frac{\text{distance of solute}}{\text{distance of solvent}}$$

The k' value in HPLC is related to the R_f value in TLC by:-

$$k' = 1/R_F - 1$$

or

$$R_f = 1/1+k'$$

The k' value measures the ratio of the time spent by the solute in the stationary phase to the time spent in the mobile phase (Pryde and Gilbert, 1979). K' can be calculated from R_F (Table 11)

TABLE 11

COMPARISON OF R_f and K' ( ) - WITH DIFFERENT SOLVENTS PROPORTIONS BETWEEN RETINOL AND ITS DERIVATIVES

Solvent \ Rf(K')	Retinol	Retinoic Acid	Retinal	Retinol Acetate
Diethyl ether	0.97(0.03)	1.0(0.0)	1.0(0.0)	1.0(0.0)
Dichloromethane	0.34(2.9)	0.07(13.3)	0.45(1.2)	0.97(0.03)
Chloroform	0.51(0.96)	0.29(2.45)	0.94(0.06)	1.0(0.0)
Acetic Acid	0.84(0.19)	0.87(0.15)	0.88(0.14)	0.84(0.19)
Dichloromethane:ethanol: ammonia 90:10:0.1	0.87(0.15)	0.46(1.17)	0.98(0.02)	-
75:10:0.1	0.97(0.03)	0.02(50.0)	1.0(0.0)	1.0(0.0)
75:1:0.1	0.43(1.3)	0.00(0.0)	0.75(0.33)	0.94(0.06)
Dichloromethane:ethanol: formic acid 90:10:01	0.91(0.1)	0.93(0.08)	with solvent front	
75:10:0.1	0.99(0.01)	0.96(0.04)	with solvent front	
75:1:0.1	0.48(1.08)	0.33(2.03)	0.8(0.25)	0.99(0.0)
10% methanol in benzene	0.57(0.75)	0.5(2.0)	0.92(0.09)	1.0(0.0)
Benzene*	0.21(3.76)	0.06(15.67)	0.36(1.78)	0.68(0.47)
Chloroform*	0.54(0.85)	0.26(2.85)	0.88(0.14)	0.98(0.02)
80:20 cyclohexane: ether	0.21(3.76)	0.23(3.35)	0.57(0.75)	0.78(0.28)
1% tetrahydrofuran: hexane	0.08(11.5)	0.04(24)	0.15(5.67)	0.28(2.57)
10% ethanol in petroleum ether	0.38(1.6)	0.42(1.4)	0.52(0.9)	0.71(0.4)

* silica gel plates used.

b) HPLC on silica column

A straight-phase (Silica column) was used to separate retinol and its derivatives. Those solvents which gave different R_F and k' (calculated as on Page 38) between retinol and its derivatives on TLC were investigated using HPLC.

Most of the solvents which were promising from TLC studies proved unsatisfactory when used in an HPLC system. However, we approached an acceptable k' value and resolution value (Rs) between retinol, retinal, retinoic acid and retinol acetate by using straight phase HPLC and Hexane: tetrahydrofuran: formic acid (93:7:0.1) proportion (Table 12). The resolution between retinal and retinoic acid had a satisfactory Rs = 1.6, whereas Rs = 0.87 was found between these compounds using Hexane: ether: formic acid (93:7:0.1). Resolution with Rs <1 is usually not successful due to overlap of peaks.

Optimised HPLC method for measurement of retinol and its derivatives

The mobile phase consisted of Hexane:tetrahydrofuran:-formic acid (93:7;0.1), at ambient temperature, with a flow rate 1 ml/min, wavelength 325 nm, chart speed 0.5 cm/min, scale expansion (absorbance settings AUFS) varied depending upon the samples injected. An example of the separation of standards is shown in Figure 11.

Extraction procedure - modification of Schade (1981) method

0-100 µl of an aqueous solution which contained a standard amount of retinol or its derivatives was transferred to C_{14}/C_{15} QQ tubes at 37°C and evaporated under oxygen-free nitrogen. 200 ul of vitamin-free serum was added and equili-

TABLE 12

COMPARISON OF K', BETWEEN RETINOL AND ITS DERIVATIVES ON STRAIGHT PHASE (HPLC), USING DIFFERENT SOLVENTS

K' / Solvent	Retinol	Retinoic Acid	Retinal	Retinol Acetate
Dichloromethane:ethanol: formic acid 75:1:0.1	0.7	1.2	1.6	1.3
Dichloromethane	1.7	1.9	1.9	0.13
Chloroform	0.0	0.0	0.05	0.05
Dichloromethane:ethanol: ammonia 75:1:0.1	0.1	0.0	0.1	0.2
10% ethanol in petroleum ether	0.23	0.41	0.11	0.06*
2:98 ether:petroleum ether°	10.8	10.6	5.8	1.2
Petroleum ether:dichloro-methane:isopropanol + 80:19.3:0.7	0.14	0.24	0.24	0.26
10% dichloromethane	0.38	0.32	0.32	0.0
1% tetrahydrofuran :hexane	0.05	0.14	0.19	0.09
Hexane:tetrahydrofuran : formic acid 93:7:0.1	4.0	0.9	0.7	0.2
Hexane:ether:formic acid 93:7:0.1	4.6	2.1	1.9	0.7

* appears with solvent peak
° not good peak shape
+ 80-100°C peteroleum ether

brated for 10 minutes. The mixture was vortexed and 200 ul of an aqueous solution of IS was added. The mixture was extracted with 1.0 ml hexane by vortexing for 5 minutes, and then centrifuged. Cyclohexane and diethyl ether were also studied. 0.5 ml of supernatant was transferred and the remainder was re-extracted with a further 1.0 ml hexane. The combined extracts were evaporated under oxygen-free nitrogen. The residue was dissolved in 100 ul hexane, eluent or cyclohexane. Table13 shows the % relative recovery of retinoic acid, retinal and retinol. The sensitivity was equivalent to 1.2 ng (0.2 µmol/l) for retinol, retinal and 6.0 ng (1.0 µmol/l) on column for retinoic acid.

These results are not satisfactory for recovery of retinol and its derivatives. The results for hexane/hexane agree with those of Schade,(1981), who achieved a reasonable recovery only of retinol and retinol acetate, with an ODS column and methanol as solvent.

Extraction method used in Western Infirmary, Glasgow

0-100 µl of an aqueous solution containing a standard amount of retinol or its derivatives was mixed well in a C14/C15 QQ tube with 0.5 ml vitamin-free serum and 100 µl of an aqueous solution of IS. The mixture was equilibrated for 10 minutes. 2 ml methanol was added, mixed, equilibrated for another 10 minutes and 2 ml distilled water was then added. 3 ml diethyl ether was added, and tubes were shaken for 15 minutes and centrifuged at 2500 rpm for 10 minutes. The ether layer was transferred to a fresh tube and the aqueous phase was re-extracted with another 3 ml diethyl ether. The ether layers were combined and evaporated to dryness at 40°C under a stream of nitrogen. Dried extracts

TABLE 13

Relative % recovery of retinoic acid, retinal and retinol from serum following extraction and solubilisation by different organic solvents.

		% Recovery		
Solvents for extraction	Solvent used to dissolve residue	Retinoic Acid	Retinal	Retinol
Cyclohexane	Cyclohexane	10	20	15
Hexane	Hexane	30	40	80
Diethylether	Hexane	30	40	20
Hexane	Eluent	27	15	-
Hexane	Cyclohexane	30	7	-
Cyclohexane	Hexane	34	15	15

were dissolved in hexane. Eluent was used also instead of methanol, which is immiscible with the mobile phase. Vitamin-free serum from the Western Infirmary was compared with freshly prepared vitamin free serum from Glasgow Royal Infirmary. (Tables 14 and 15).

Better results were obtained with the vitamin-free serum prepared in Glasgow Royal Infirmary because the serum used by the Western Infirmary gave a peak of retinol with our method. They used reconstituted dried plasma in preparing vitamin-free serum. These recoveries are however still unsatisfactory.

Extraction method of Ito, et al (1974)

The method had 4 steps. The plasma was lyophilized and extracted twice with chloroform:methanol (1:1) containing 50 µg of BHA/ml. Then the precipitate was re-extracted twice with methanol containing 50 µg of BHA/ml. The supernatants were combined, evaporated, and the residue was dissolved, and injected onto the column.

The results seem satisfactory with lyophilization and full extraction for both % relative recovery and absolute recovery ( ) for retinol, retinoic acid and retinol acetate if compared to Ito et al (1974) (Table 16). The procedure was too long so a modification was done by a reduced extraction to one step chloroform: methanol (1:1) and with no lyophilization (Table 17).

The results show a good satisfactory % relative and absolute recovery for retinol, retinoic acid and retinol acetate, while poor recovery for retinal. However, a satisfactory separation of all derivatives was achieved. Although the

TABLE 14

Comparison of absolute and relative % recovery of retinoic acid retinal and retinol between Western and Royal Infirmary methods (Glasgow)

Compounds	Western Infirmary Serum % absolute recovery	Royal Infirmary serum	
		% Absolute Recovery	% Relative Recovery
Retinoic acid	100%	100%	90%
Retinal	27%	23%	58%
Retinol	50%	78%	65%

TABLE 15

Relative % recovery of retinoic acid, retinal and retinol following solubilisation by different organic solvents.

Compounds	% Relative recovery	
	Hexane	Eluant
Retinoic acid	95	7
Retinal	58	29
Retinol	53	-

TABLE 16

Relative and absolute % recovery( ) of retinol, retinoic acid, retinol acetate and retinal using Ito et al (1974)

Solvent	Ito et al (1974) (Retinol)	Retinol	Retinoic Acid	Retinol Acetate	Retinal
2xChloroform:methanol (1:1)	89.4	62 (75)	71 (77)	(77)	-
2 x Methanol	9.9	9 ( )	-	-	-
Full extraction	99.3	70 (82)	98 (90)	(95)	27 (29)

TABLE 17

Relative and absolute % recovery ( )for retinol, retinoic acid, retinol acetate, and retinal after modification of Ito et al (1974)

Solvent	Retinol	Retinoic Acid	Retinol Acetate	Retinal
1 x Chloroform:methanol (1:1)	90(100)	97(97)	-(90)	30 (36)
Full Extraction	88(92)	96(88)	-(90)	40(25)

recovery of retinal was low, it is still adequate to detect and quantitate retinal in serum. Accurate quantitation of retinal would require improved recovery.

No difference was found in retention time, k' or recovery if air is used instead of nitrogen to dry the residue (Figure 12).

Solubilizing solvent selection

Different solvents were used to dissolve residues of retinol and its derivatives after extraction. The k' was measured (Table 18).

Therefore cyclohexane, dichloromethane, and chloroform could all be used, but we chose the mobile phase as the solvent to dissolve residue because it gave good R_S between retinal and retinoic acid, R_S = 1.6. Other agents failed due to interference with solvent peak, or internal standard. The immiscibility of some give an artifact peak or produced high pressure.

Preparation of standard curve for HPLC analysis of retinol and its derivatives

0-150 µl of retinol and its derivatives were dried in C14/C15 QQ tubes with nitrogen. 200 µl of vitamin-free serum was added and equilibrated for 10 minutes. 50 µl of internal standard was added, mixed, then 1 ml of chloroform; methanol (1:1) containing 50 ug butylhydroxieanisolewas added, shaken for 15 minutes and centrifuged. The upper layer was transferred to empty tubes and dried with air. The residue was dissolved in 100 µl of mobile phase used for HPLC, vortexed for 15 seconds and 20 µl injected manually on the column.

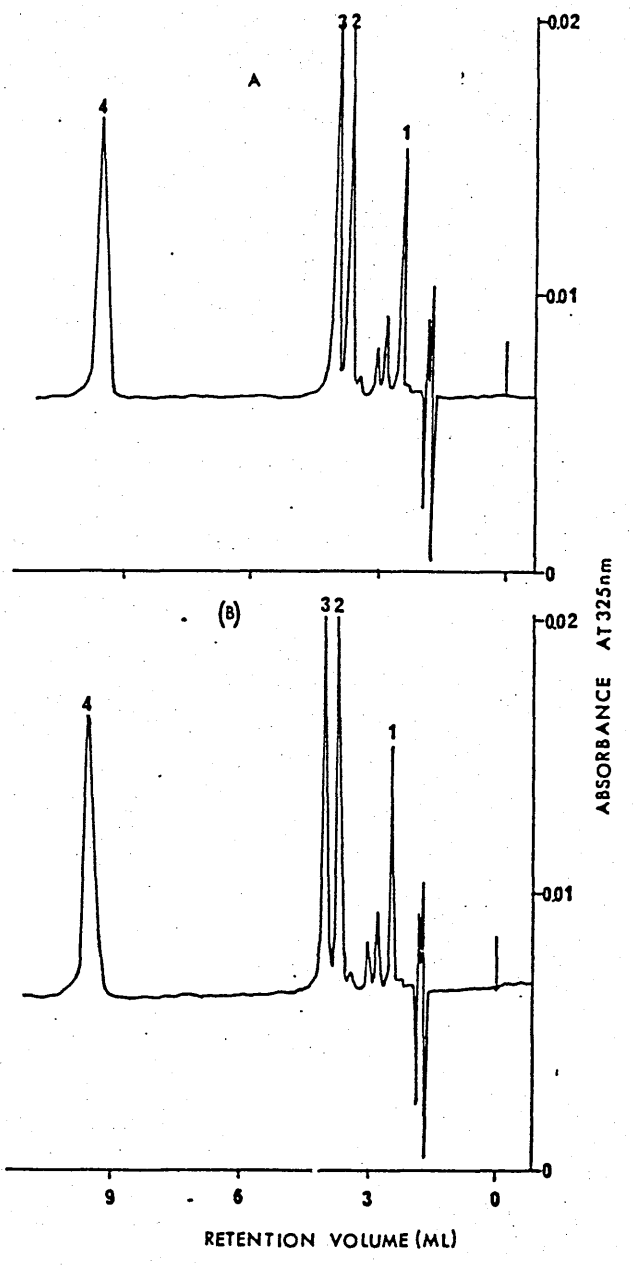


Figure 12: Chromatography of retinol and its metabolites drying by air (A) and nitrogen (B)

Peak identities as in Figure 11.

TABLE 18

Comparison of k', between retinol and its derivatives, to find a good organic solvent solubilizer

organic solvent \ k'	Retinol	Retinoic Acid	Retinol Acetate	Retinal
Acetone	N:L	N:L	N:L	N:L
Acetonitrile	Immiscibility with mobile phase			
Methanol	"	"	"	"
Ethylacetate	High pressure			
Tetrahydrofuran (THF)	"	"		
Cyclohexane	5.2	1.2	0.28	0.94
Dichloromethane	5.1	1.2	0.28	0.90
Chloroform	4.7	1.0	0.16	0.8
Eluent (mobile phase) [93:7:0.1 (H;THF:F.ac]	4.3	1.3	0.35	1.1

N:L = no peak

Linearity and sensitivity

The linearity was determined as a function of on column sample weight. 20 µl aliquots of aqueous solution of retinol over a concentration range of 0-4.5 mg/l (0-15.7 µmol/l) equivalent to 0-90 ng on column weight were studied.

The assay for retinol was linear over the range 0-90 ng on column sample weight for extraction from serum with internal standard or for directly injected aqueous standard equivalent to 0-4.5 mg/l (0-15.7 µmol/l).

Similarly for retinoic acid linearity over a range of 0-60 ng/ml equivalent to 0-3 mg/l (0-10.5 µmol/l) was found.

The lowest detectable serum concentrations (defined as a signal to noise ratio of 2) was 60 µg/l (0.2 µmol/l) for retinol and 50 µg/l (0.17 µmol/l) for retinoic acid. The sensitivity was equivalent to 1.2 ng retinol and 1.0 ng on column for retinoic acid.

Recovery

Solutions of retinol and retinoic acid in serum covering the range of 0-3 mg/l (0-10.5 µmol/l) were analysed. The difference in detection response between the extracted and directly injected aqueous standard represented the absolute percentage recovery (Table 19). Sera of known vitamin A concentrations (prepared from vitamin-free serum) to which internal standard had been added were assayed and the results compared with calibrations to represent relative recovery.

TABLE 19

Absolute %-recovery of retinol, retinoic acid and retinal from serum (n = 20)

Compound	% Absolute Recovery		
	$\bar{x}$	±SD	%CV
Retinol	91.5	2.3	2.6
Retinoic Acid	88	2.8	3.3
Retinal	36	3.6	10.0

The relative recovery of retinoic acid was 93 ± 5.7%, retinol was 95.3 ± 3.7% and retinal 25.7 ± 3.7%. Recovery of retinol and retinoic acid was satisfactory, and although that of retinal was poorer, it was considered adequate for further studies.

Precision

The precision was assessed by determining retinol and retinoic acid in 10 serum extracts at each of 3 different concentrations. Within-batch as well as between-batch studies were performed (Table 20).

Precision was considered satisfactory over the concentration range for retinol and retinoic acid, for the within-and between-batch samples. All except retinoic acid fell below 6% CV.

Accuracy and specificity

The possibility of interference by a number of drugs especially those taken by patients during treatment was checked by the addition of aqueous solution of drugs to vitamin-free serum. The drugs studied were Zinacef, Temazepam, Nitrazepam, Tagamet, Amoxil, Flagyl, Genticin , Kefzol, Ampicillin, Nebcin, Ceporin, Kannasyn, Penicillin, Cefotaxime , Kefzol , Librium, Chlorazapate , Ceporex, Oxazepam, Amikin,Clonazapam, Medazepam, Diazepam, Gentamicin, (vials and powder). 20 µl samples were then analysed on the HPLC column for peaks coinciding with the retention time of retinol and its analogues.

Some of those drugs showed peaks eluting late in the analyses. None of those drugs examined interfered with the assay of retinol and its derivatives.

TABLE 20

Precision studies on retinol and retinoic acid in serum (n = 10)

Compound	Concentration Mass units	Molar units	CV % within-batch	CV % between-batch
Retinol	0.7 mg/l	2.4×10^{-6}	3.9	4.2
Retinol	1.5 mg/l	5.2×10^{-6}	3.8	4.1
Retinol	3.0 mg/l	1.1×10^{-5}	2.6	2.3
Retinoic acid	0.56 mg/l	1.9×10^{-6}	4.6	5.1
Retinoic acid	1.5 mg/l	5.0×10^{-6}	4.4	6.6
Retinoic acid	3.0 mg/l	1.0×10^{-5}	6.1	5.6

2.1.4 Comparison of HPLC and fluorometric methods for retinol analyses

Sera from 70 healthy fasting adults were analysed by fluorometric as well as HPLC methods and the correlation was plotted (Figures 13 and 14).

A poor correlation of ($r = 0.7$) was found, which agrees with the results of De Ruyter and De Leenher, (1976). In general, it was found that the HPLC method gave higher results than fluorimetry.

Discussion

Considerable difficulty was found in developing a method of separating retinol, retinal and retinoic acid in serum, whilst using retinol acetate as internal standard (IS).

The HPLC described is highly sensitive and linear over a wide range. Other workers (Table 5) found that their HPLC procedure was less sensitive and linear over only 30% of our procedure and there was poor calibration for their method because vitamin-free serum was not used. Although De Ruyter and DeLeenher (1976) succeeded in separating retinol and its derivatives, they failed with retinoic acid, which had a $k' = 5.2$ and might interfere with retinol ($k' = 5.0$).

The fluorimetric method is less sensitive and linear over only 20% of the range covered by the HPLC procedure, whereas the accuracy and sensitivity of the HPLC assay makesthe use of the technique preferable to the fluorimetric method.

The fluorimetric procedure may be preferred where rapid results are required. However, for pharmacokinetic studies it is necessary to measure the unchanged drug and its major metabolites

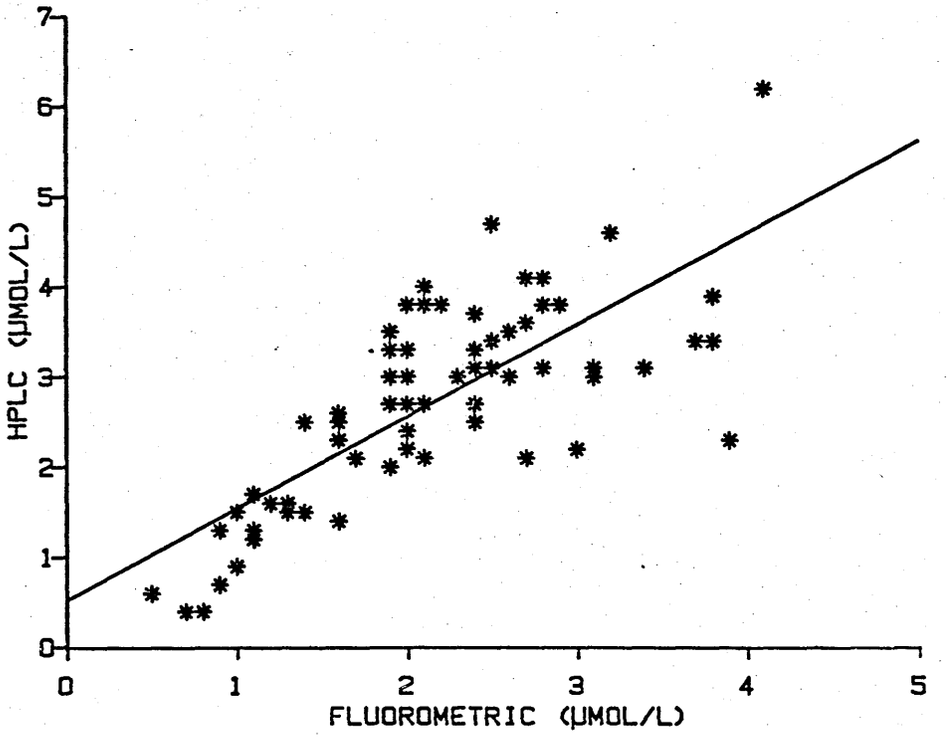


Figure 13: Correlation between serum vitamin A concentration measured by fluorometric and HPLC methods in control patients

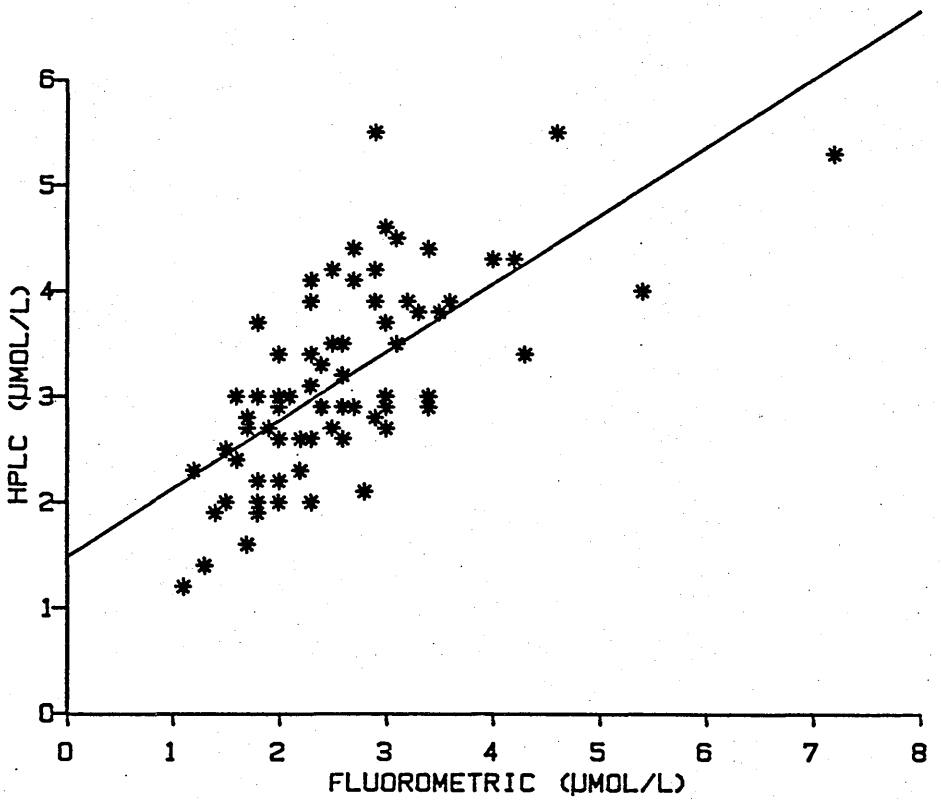


Figure 14: Correlation between serum vitamin A measured by fluorometric and HPLC methods in cancer patients.

in serum. The HPLC method generally gave higher results than the fluorimetric. This is surprising in view of the lack of specificity of the fluorimetric method, since other compounds were also detected. The possible reasons for this were: firstly, quenching may have occurred due to haemolysis or other substances in serum, which interferes with the fluorimetric method. For example, a control sample [(Clinical Chemistry QC (adjusted Bovine serum)] Rdien Fraction Centre was measured by both HPLC and fluorometric method F = 0.9 ± 0.2 µmol/l and HPLC = 2.3 ± 0.1 µmol/l. There are peaks other than retinol (Figure 15) by HPLC, which may have led to quenching in the fluorometric method.

Secondly, in the fluorometric method, if retinol is used instead of retinol acetate as standard, better results are obtained (Section 2.1.2.3) due to more effective extraction of retinol than retinol acetate.

Thirdly, retinol and its esters are the only retinoids that fluoresce appreciably under normal conditions. Anhydro-retinol, retinoic acid and retinaldehyde do not fluoresce in conventional assay procedures but may do so at very low temperatures or under special conditions (Frolik and Olson 1984). So the fluorometric method measures retinol only and ester forms present and not total retinoids as was thought before. In experiments carriedout on "nutrition" patients (Section 3.2.3) retinol palmitate measured by HPLC gave higher results than fluorometry which measures total retinol and ester form. There may therefore be a saturation concentration for the fluoro-metric method at which an increase in concentration may not lead to an increase in fluorescence.

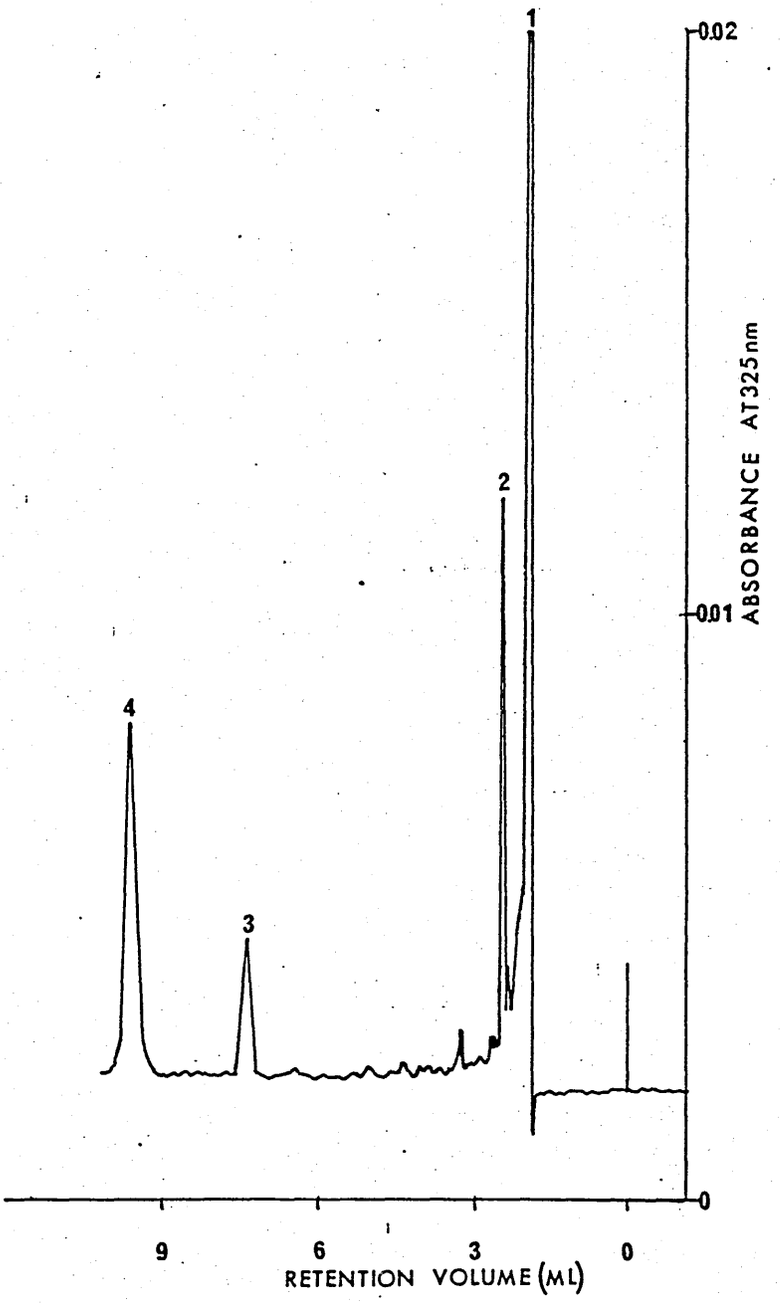


Figure 15: HPLC chromatograph of bovine serum control. Peak identities are:-

1 - carotene
2 - retinol acetate (IS)
3 - unidentified peak
4 - retinol

Kahan, (1966) found a linear relationship between fluorescence and concentration within the range of 0.2 - 300 µmol/l. The deviation at levels higher than 300 µmol/l is due to concentration quenching.

Phytofluene occurs in common fruits and vegetables and the levels in blood vary considerably, reflecting the consumption of foods such as tomatoes. The error introduced into the determination of vitamin A varies erratically from 20 to 200% and the mean elevation has been reported to be 30 µg/100 ml. (Thompson et al 1973). Because of this a correction formula was used in earlier methods.

Experience in this laboratory indicates that in order to produce acceptable within and between-batch precision, all extraction procedures should employ an internal standard.

Many workers referred to before did not use an internal standard. The retinol acetate used in this study as IS exhibited similar chemical behaviour to retinol and was not found in any sera analysed. No previous workers mentioned the occurrence of this derivative in serum. It can be resolved from retinol and other metabolites on the HPLC column with no interference and is also commercially available.

The method presented here gives a simple reliable extraction procedure for the rapid determination of retinol and retinoic acid in serum. It also separates carotene and retinal from these substances but with poor recovery for retinal. This method could therefore be used to determine if retinal is present but if so a further quantitative method would be

necessary. So far as is known, the method developed during this present study is unique in separating retinol and its derivatives in one chromatogram.

2.1.5 Development of an HPLC method for retinol palmitate

Previous workers used different methods to estimate retinol palmitate, and in this present work we used two methods, either direct HPLC analysis or saponification followed by analysis of total retinol.

Allwood (1982) used HPLC with 10 µODS column and ultraviolet (UV) detector at 325 nm. He succeeded in analysing retinol palmitate from TPN solution over a concentration range of 0.75-3 µg/ml.

Kishi et al (1981) used HPLC to determine the stability of retinol palmitate in TPN solution, using Hitachi Gel 3011 (4 mm x 250 mm) column with UV detector at 254 nm.

Dahl (1982) used a reversed phase column (5 µODS) with detection at 254 nm. He succeeded in determining retinol palmitate in adults as well as infants receiving Vitlipid^{R} (Kabi Vitrum).

Finally, Hartline and Zachman (1976) analysed retinol palmitate by a modification to the Needld-Pearson technique (1963) using trifluoroacetic acid reagent with hexane extraction in place of petroleum ether.

Most of these methods succeeded in analysing retinol palmitate. They did not however mention interference of the other vitamin A derivatives, due to the fact that if these infusions are exposed to light, decomposition of retinol palmitate occurs. Allwood, (1982) mentioned the effect of trace

elements as well as amino acids on vitamin A in TPN solutions. For analysis of retinol palmitate a minor change in the polarity of mobile-phase is needed to the HPLC method we used to analyse serum retinol and other derivatives. We could therefore use only one system with a switch in polarity for analysis of all retinol metabolites.

Direct HPLC analysis of retinol palmitate

Materials and Apparatus

Intralipid^{R} (Kabivitrum, Stockholm, Sweden); 500 ml 20% fat emulsion for intravenous use containing 100 g fractionated soya bean oil, 6 g fractionated egg phospholipids, 11.25 g glycerol and water for injection to 500 ml (osmolality 350 mosmol/kg water); Vitlipid^{R} (Kabi Vitrum), 'adult' 10 ml was added aseptically. It is a sterile emulsion in the oil phase of an emulsion the same composition as Intralipid; 1 ml of it contains retinol palmitate (75 µg), vitamin D (0.3 µg) and vitamin k_1 (15 µg). Potassium hydroxide, sodium sulphate, anhydrous sodium sulphite, isopropanol (BDH Chemicals Ltd, Poole, England) and other materials as in Section (2.1.3.2).

Apparatus

As described in the previous section for serum retinol investigation.

Methods

Polarity Investigation

Hexane:tetrahydrofuran:formic acid (93:7:0.1) was first used, and the polarity was changed by altering the ratio of hexane to tetrahydrofurane. K' of the metabolites were studied.

In general, retinol palmitate eluted with the solvent peak. The retention time of retinol palmitate was not altered at most polarities (Fig 16). Only with a ratio of Hexane:tetrahydrofuran :formic acid (99:1:0.1) was a successful separation of retinol derivatives obtained (Fig 17 Table 21).

Optimised Methods for Direct HPLC analysis of retinol palmitate

The mobile phase consisted of Hexane:tetrahydrofuran :-formic acid (99:1:0.1). The operating conditions were; temperature ambient. Flow rate 1 ml/min, wavelength 325 nm, chart speed 0.5 cm/min Absorbance setting varied depending upon the samples injected.

Saponification of retinol esters

Modification of van de Weerdhof et al (1973)

Nutritional emulsion (Intralipid) as well as standard amounts of retinol palmitate and retinol individually were added (0-6 ml) to a 250 ml round bottomed flask with 50 ml 0.5 N potassium hydroxide in ethanol and 1 ml of 10% sodium sulphite (Na_2SO_3) solution. After refluxing for 30 min, the mixture was cooled in ice, 100 ml Hexane was added and the mixture was transferred into a separating funnel containing 50 ml of 1N potassium hydroxide solution and shaken for 10 sec. After separation the water layer was rejected. The hexane layer was washed with 50 ml 1N potassium hydroxide solution and subsequently three times with 50 ml of distilled water, and then dried with sodium sulphate (Na_2SO_4). It was then filtered, and 20 µl of filtrate was injected on to the HPLC column.

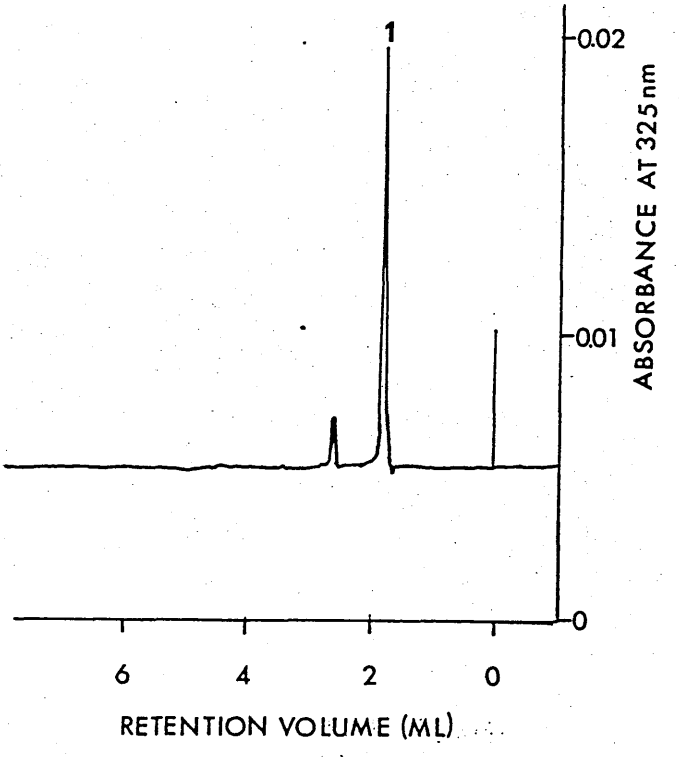


Figure 16: HPLC chromatograph showing retinol palmitate standard (1), eluted with solvent peak with different preparation of mobile phase eg

93:7:0.1	Hexane:THF:Formic acid
95:5:0.1	Hexane:THF:Formic acid

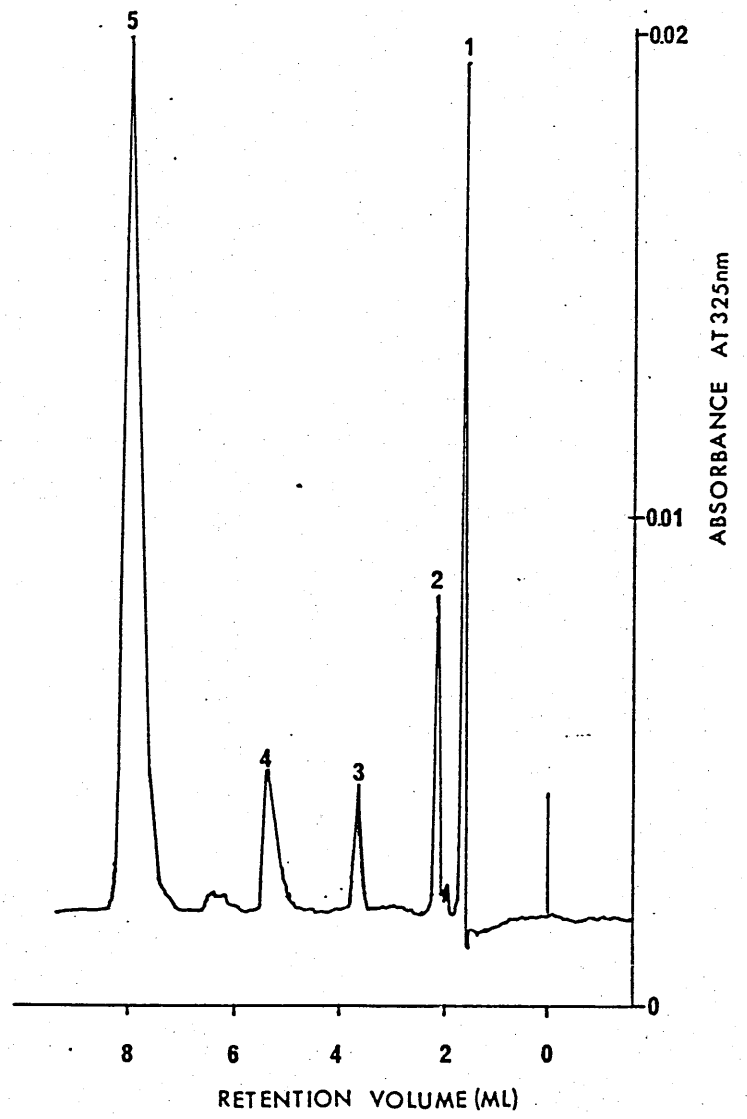


Figure 17: Chromatograph of vitamin A metabolites and standard with mobile phase preparation of 99:1:0.1: H:THF:F.ac, peak identities are

1 - carotene
2 - retinol palmitate
3 - retinal acetate (IS)
4 - retinal
5 - retinoic acid

Retinol was eluted at about 60 mins

TABLE 21

Study of k', of Retinol and its derivatives following elution with Hexane:THF:Formic acid 99:1:0.1 proportion

k'	Carotene	Retinol palmitate	Retinol acetate	Retinal	Retinoic Acid	Retinol
k'	0	0.35	1.3	2.4	4.0	17.1

Modification of saponification procedure

Because all retinol palmitate was not hydrolysed using the above method (Figure 18)

1) Increasing the saponification times up to 2 hour, and duration of shaking to 1 hour respectively was performed. The concentration of potassium hydroxide (KOH) was increased from 0.5 N to 5 N KOH, but these changes did not improve efficiency of hydrolysis.

2) Standard retinol palmitate stock solution was prepared in methanol as well as water.

3) Since retinol was eluted very late with retinol palmitate HPLC method, Hexane:tetrahydrofuran:formic acid (93:7:0.1) proportion was used for retinol analysis following saponification.

Recovery and Linearity

Either retinol palmitate or retinol was added to Intralipid 20% emulsion covering the range of 0-2.5 mg/l (4.8×10^{-6}M). The mixture was saponified and analysed for retinol. Results were compared with direct injection of retinol standard. Results represent the percentage absolute recovery. The theoretical calculation of conversion of retinol palmitate to retinol is:-

Retinol palmitate ⟶ Retinol + palmitic acid

1 mmol ⟶ 1 mmol

524.5 (MW) ⟶ 286.5 (MW)

Therefore 100 µg retinol palmitate produces 55 µg retinol after saponification.

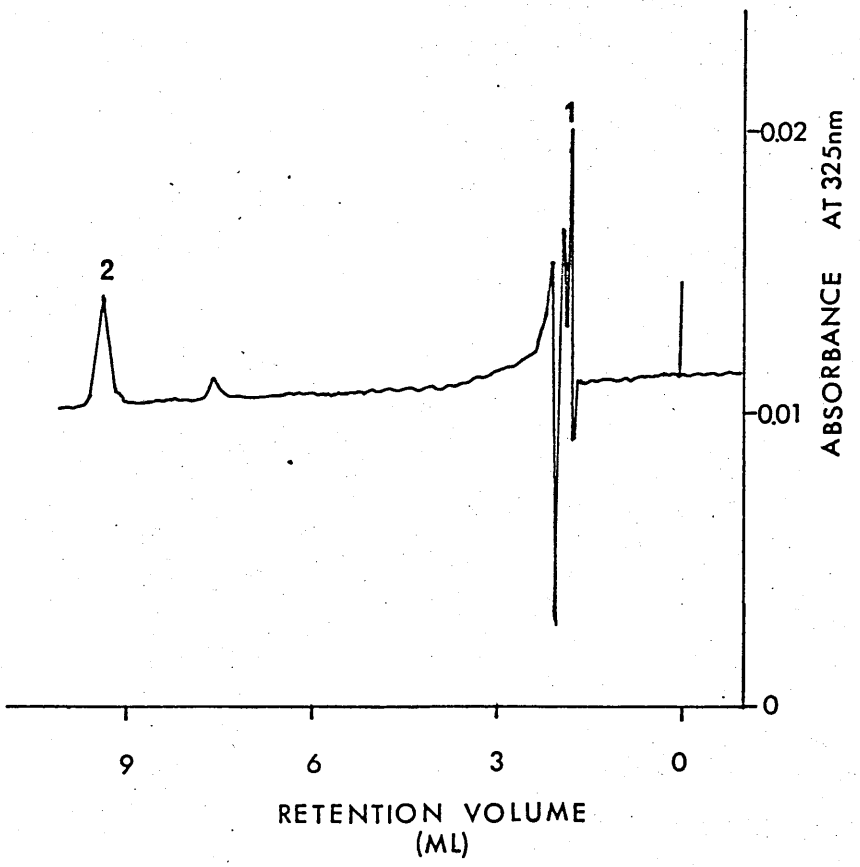


Figure 18: HPLC chromatograph of Intralipid emulsion after saponification. The mobile phase was 93:7:0.1 (Hexane:THF:Formic acid) preparation. Peak identities are:-

1 - retinol palmitate in solvent peak
2 - retinol.

Linearity was determined as a function of on-column sample weight by chromatographing 20 µl aliquots of the saponified solution of retinol palmitate over a concentration 0-2.5 mg/l (0-4.8 x 10^{-6}M) equivalent to 0-50 ng on-column weight.

Percentage absolute recoveries of retinol palmitate from standard solutions after saponification were 101% in methanol, and 64% in water (Table 22).

Thus, this method is satisfactory for standards and also for liver analysis (Section 2.1.6), but is not suitable for analysis of fat emulsions.

2.1.5.1 Extraction of retinol palmitate from serum

The method optimised in Section 2.1.3.2 for extracting retinol from serum was used here to measure recovery of retinol palmitate after extraction from serum and nutritional solutions. At the same time linearity and precision were studied, as well as sensitivity.

The chloroform:methanol extraction failed to extract retinol palmitate fully from nutrition solution (Figure 19) due to the presence of large amounts of lipid and two layers formed. However, the percentage absolute recovery was measured for standard retinol palmitate from serum extraction and was compared with direct injection on the same amount of retinol palmitate in Hexane:tetrahydrofuran:formic acid (99:1:0.1). The absolute recovery was 92±1.5% ($\bar{x}$ ± 1SD) and linear over a range 0-0.4 µg/column (0-38 µmol/l). Sensitivity, as the lowest detectable concentration was 0.1 µmol/l. Precision for three different concentrations is shown in Table 23.

TABLE 22

Absolute % recovery of retinol palmitate standard, after saponification and dissolving in Methanol and water.

Solvent	% Retinol palmitate after saponification	% Retinol palmitate expected	% Recovery
Methanol	56%	55%	101%
Water	33%	55%	64%

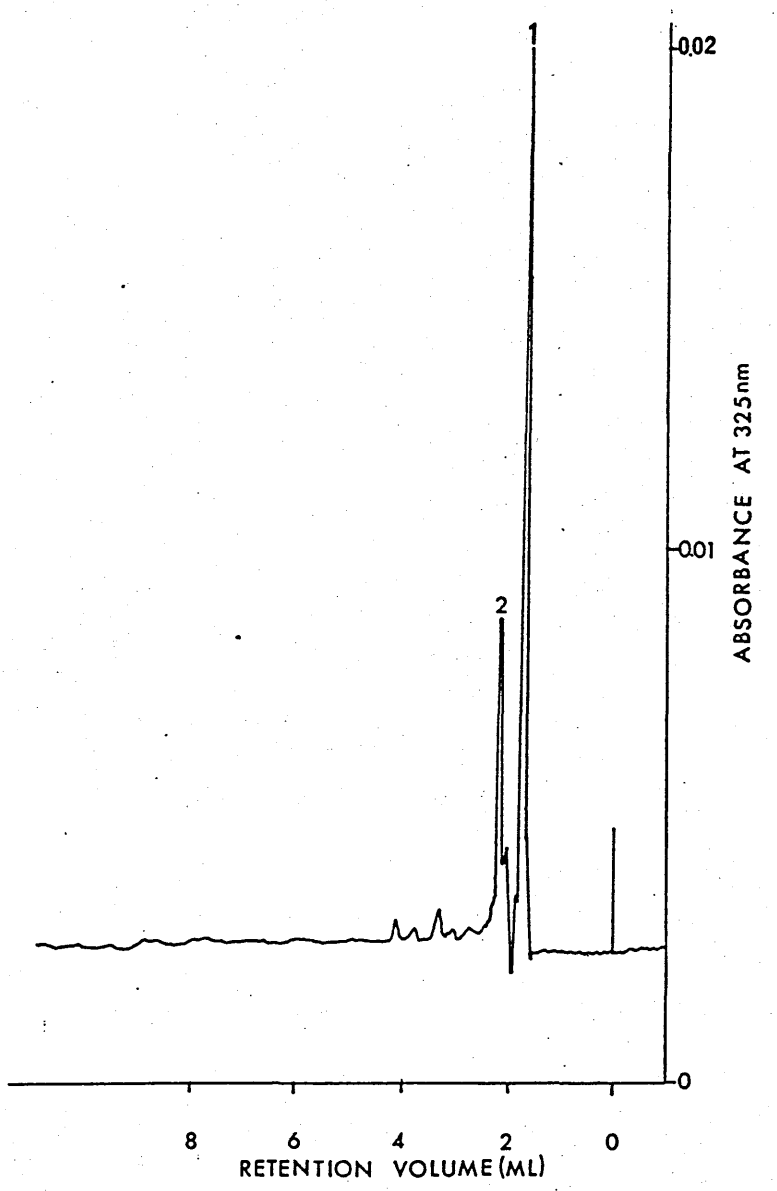


Figure 19: Chromatograph of Intralipid emulsion after extraction with methanol:chloroform (1:1) preparation.

Peak identities are:-

1 - unidentified peak in solvent peak
2 - retinol palmitate

TABLE 23

Precision studies on retinol palmitate analysis in serum (n = 10)

Mass Units	Molar Units	% CV
200 µg/l	4.9×10^{-7}	5.0
600 µg/l	1.1×10^{-6}	3.7
1.8 mg/l	3.4×10^{-6}	1.5

2.5.1.2) Measurement of retinol palmitate in fat emulsion (Dahl 1982)

The amount of retinol palmitate in Intralipid emulsion was determined by HPLC, by dissolving the sample in isopropanol and injecting directly on the column.

1) An accurate amount of retinol palmitate standard was weighed (20 mg) and diluted with isopropanol solution to 100 ml (solution 1).

2) 1 ml was pipetted from solution 1 and diluted to 100 ml with isopropanol as a working standard (solution 2).

3) A standard curve for three solutions was prepared using 5, 10 or 15 ml from solution 2 diluted to 50 ml with isopropanol. This was directly injected on the column.

4) A standard curve for extraction from fat emulsion was prepared by pipetting 1 ml from solution (1), plus 10 g of Intralipid 20% emulsion (with no Vitlipid) which was diluted to 100 ml with isopropanol. From this solution three different standards were prepared as in No. (3), diluted with isopropanol. (Test injection I). Three different standards were prepared and diluted with 25.0g Intralipid 20%, diluted to 250 ml with Isopropanol) instead of isopropanol. A further 1:2 dilution was also made.

5) Analysis of test emulsion was carried out by measuring 1 g of the emulsion diluted to 10 ml with isopropanol. To assess recovery standard was added to the unknown

emulsion. (3). The absolute recovery, linearity, sensitivity and precision for four concentrations were studied.

Linearity and sensitivity:

The linearity was determined as a function of on-column sample weight by chromatographing 20 µl aliquots of retinol palmitate over a concentration range 0-2.5 mg/l (4.8×10^{-6} M) equivalent to 0-50 ng on column weight.

The assay for retinol palmitate was linear over the range 0-50 ng on-column sample weight for extraction of Intralipid solutions or for direct injection of standard equivalent to 0-2.5 mg/l (0-4.8×10^{-6}M).

The lowest detectable concentration (defined as a signal to noise ratio of 2) was 30 µg/l equivalent to 0.6 ng on column weight.

Recovery

Solutions of retinol palmitate in Intralipid covering the range 0-2.5 mg/l (0-4.8×10^{-6}M) were analysed. The difference in detector response between the standard with Intralipid solution and directly-injected aqueous standard represented the absolute recovery (Table 24).

Precision

The precision of analysis was assessed by determining retinol palmitate in 12 solutions each of 3 different concentrations 0.2 mg/l, 0.4 mg/l and 0.6 mg/l equivalent to 4.0×10^{-7}, 8.0×10^{-7} M, 1.1×10^{-6} M) respectively (Table 25).

TABLE 24

Absolute % recovery of retinol palmitate from TPN solution (n = 30)

Mass Units	% ($\bar{x}$)	± SD	%CV
0.4 (mg/l)	97.7	± 2.8	2.9

TABLE 25

Precision studies on retinol palmitate in TPN solution (n = 12)

Mass Units	Molar Units	% CV
200 µg/l	4.0×10^{-7}	3.1
400 µg/l	8.0×10^{-7}	2.9
600 µg/l	1.1×10^{-6}	3.2

2.1.5.3) Estimation of retinol palmitate in liver

Most of the previous workers estimated retinol in liver by saponifying retinol esters to retinol and measuring the total.

Olson et al (1979) hydrolysed retinol palmitate in liver by grinding with anhydrous sodium sulphate and leaving the mixture overnight under chloroform. Aliquots of the chloroform extract were then analysed either by spectrophotometric or Carr-Price assay. Both methods generally agreed within 8%.

Flores and Aranjo (1984) estimated vitamin A in liver, by homogenising the liver samples with 50 vol of 50% glycerol in water. Retinol was assayed spectrophotometrically in extracts of the non saponifiable lipids of the homogenates.

Table 26 shows some methods of extraction and estimation of vitamin A in liver.

Most of the methods measured total vitamin A in the liver. In our studies we attempted to estimate retinol palmitate, retinol and total vitamin A by three different methods.

Sample preparation for retinol palmitate measurements (Bhat & Lacroix, 1983)

The specimen of liver was washed with cold phosphate-buffered saline and lyophilized overnight. The lyophilized liver was ground to a powder with a pestle and mortar and extracted with 20 ml 99% methanol per gm tissue and then with 50 ml of hexane per gm tissue. The methanol and hexane extracts were evaporated separately and the residues were combined after redissolving in Chloroform:Methanol (1:1). An aliquot was taken and injected on HPLC.

TABLE 26

PUBLISHED METHODS FOR ESTIMATING VITAMIN A IN LIVER

Author	Method of Hydrolysis	Estimation of retinol	Normal Values (µg/g)
Underwood et al (1970)	Saponification with KoH Bieri (1968)	Trifluoroacetic acid Neeld and Pearson (1963)	100 - 300 µg/g wet weight
Raica et al (1972)	Hydrolysis with sodium sulphate and ether extraction. Ammes (1954)	1) Microcolumn technique. McLaren et al 1967 for retinol and retinol esters estimation 2) Trifluoroacetic acid for vitamin A analysis	1 - 4,400 µg/g accidental death 10 - 35 µg/g Retinol 18 - 1,353 µg/g retinol esters
Smith and Malthus (1962)	Saponification with KoH Moore (1937)	Beckman model DU spectrophotometer (312.5, 326.5, and 336 mU)	0 - 900 µg/g (333 µg/g)
Amedee-Manesme et al (1984)	Sodium sulphate and methylene chloride Olson (1979)	HPLC with UV detection	0 - 76 µg/g retinol 0 - 325 µg/g retinol esters 0.9 - 400 total vitamin A

1) The procedure was standardised by adding a known amount of retinol palmitate to 1 g of liver tissue from vitamin A-deficient rats (Section 3.3.2.1) and taken through the entire procedure of lyophilization, extraction and estimation on HPLC.

2) The residue was dissolved in 10 ml/g tissue eluant before injection.

Results

Absolute recovery of the above method was 62% (58-65%) but if a standard was dried.down under nitrogen then added to the homogenized liver, lyophilized and extracted, absolute recovery was 76% (70-80%) for a concentration range 0-36 ng on-column. If vitamin C and EDTA was added before lyophilization, recovery was 73% (70-75%) when another solvent was used instead of methanol/hexane to extract tissue, eg isopropanol, or mobile phase (H:THF:Fac) proportion of (99:1:0.1), the mean recovery was 70% and 75% respectively. These recoveries were regarded as unsatisfactory, the losses probably being associated with the lyophilisation.

Modified method, without lyophilisation

This procedure was standardised by adding a known amount of retinol palmitate to several mg of liver tissues equilibrated 10 min, homogenized, extracted with hexane:tetrahydrofuran:formic acid (99:1:0.1), shaken for 15 min and centrifuged at 2000 rpm. The supernatant was dried with air at 37°C. The residue was dissolved in a known amount of mobile phase, and 20 µl was injected on column. Dried liver can be used if the liver amount is small and treated as above.

HPLC Method

Hexane:tetrahydrofuran :formic acid (99:1:0.1) was used as mobile phase on a silica column for estimation of retinol palmitate.

Recovery

A solution of retinol palmitate covering the range 0-54 mg/l (0-2.7 mg/g) was analysed. The difference in detector response between the standard and the directly-injected aqueous standard solution represents absolute recovery. The mean recovery of 30 samples was 101.3 $\pm$ 2.4% with a CV of 2.4%.

Linearity

The linearity was determined over a range of 0-54 mg/l (0-2.7 mg/g) equivalent to 0-1 µg on column. The assay for retinol palmitate was linear over the range, for extraction from homogenate solution or for direct injection of aqueous standard equivalent to 0-54 mg/l.

Precision

The precision was assessed by determining retinol palmitate in 10 liver extracts at each of 3 different concentrations 300 µg/g, 600 µg/g and 900 µg/g, which were prepared from the liver of vitamin A-deficient rats by addition of standards (Table 27).

TABLE 27

PRECISION STUDIES ON RETINOL PALMITATE IN LIVER (n = 10)

Measured retinol palmitate in liver	CV %
300 µg/g	4.9
600 µg/g	1.7
900 µg/g	1.5

2.1.6) Estimation of total retinol in liver (Van de Weerdhof, et al 1973

This was performed by saponification followed by measurement of total retinol.

Mean absolute recovery of added standard was 99.8% and linearity for retinol palmitate was satisfactory over a range of 0-120 ng on-column weight (0-6 mg/g wet tissue).

Precision

The precision was determined by measurement of three different concentrations of retinol palmitate after saponification. (Table 28).

2.1.7) Estimation of free retinol in liver.

The method was similar to that for serum retinol (Section 2.1.3.2) except that 5 ml of chloroform:methanol (1:1) was used instead of 1 ml and 20 µl was injected. Dried liver can also be used.

Recovery

Solutions of retinol covering the range 0-4.5 mg/l (225 µg/g) were added before homogenisation, the difference in detector response between the standard and the directly-injected aqueous solution representing absolute recovery. The mean recovery of 20 analyses was 86.7% + 3.6 with a CV of 4.1%.

Linearity

The linearity was determined over a range of 0-90 ng on-column weight equivalent to 225 µg/g.

The assay was satisfactory over that range.

Precision

The precision was assessed by determining retinol in 10 preparations at each of three different concentrations, 37.5 µg/g, 75 µg/g and 150 µg/g (Table 29).

Discussion

The major vitamin A derivative found in Intralipid emulsion containing Vitlipid (fat soluble vitamins) as well as in liver was retinol palmitate. To follow changes of vitamin in these solutions or tissues it was therefore necessary to develop a method for analysis of this metabolite.

Van de Weerdhof et al (1973) estimated vitamin A in food by converting the ester forms to retinol by saponification. A modification of this method was used in this study, with application of the high performance liquid chromatography method used for serum retinol measurement.

When the standard was analysed by this method it gave high sensitivity and linear results over a wide range and with virtually 100% absolute recovery when dissolved in alcohol and 64% when dissolved in distilled water. The recovery from Intralipid emulsion was poor using this method. This method is therefore satisfactory to measure total retinol, which is produced from esters after saponification. For estimation of stability and delivery of retinol palmitate in this emulsion after exposure to light, and investigation of the appearance of any other compound during exposure to light, a more specific method is needed.

TABLE 28

PRECISION STUDIES ON TOTAL VITAMIN A IN LIVER (n = 10)

Measured total vitamin A in liver	CV %
2 mg/g	3.6
4 mg/g	2.3
6 mg/g	1.9

TABLE 29

PRECISION STUDIES ON FREE RETINOL IN LIVER (n = 10)

Measured free retinol in liver	CV %
37.5 µg/g	4.7
75 µg/g	4.2
150 µg/g	4.1

An investigation was carried out to find a method for extraction and estimation of retinol palmitate in this emulsion. The hexane:tetrahydrofuran:formic acid proportion of (99:1:0.1) was the best mobile phase to elute retinol palmitate. This solvent, which is less polar than that used in the HPLC method for serum retinol estimation was found to be satisfactory in terms of sensitivity, and absolute recovery. Around 100 samples can be measured per day, because each sample takes only 5 minutes to elute.

Retinol palmitate could be estimated in serum also, with an absolute recovery of 92 $\pm$ 1.5% and the procedure was sensitive and linear over a wide range. This method can therefore be used to follow clearance of retinol palmitate from serum following oral or intravenous delivery.

Bhat and Lacroix (1983) extracted retinol palmitate and other esters from liver after lyophilisation and quoted approximately 85-95% recovery, whereas we only obtained a mean of 76% recovery using this method.

An investigation was carried out to find a good HPLC method for estimation of retinol palmitate in liver. The HPLC method described earlier gave a good absolute recovery 101.3 $\pm$ 2.4% and was highly sensitive and linear over a wide range (0-2.7 mg/g). Although this method gave a separation of all the retinol derivatives, the retinol peak eluted very late (60 min after injection). It was therefore necessary to use a separate method to measure retinol in liver extracts.

Van de Weerdhof et al (1973) method was used to estimate total vitamin A and the serum retinol procedure was used to estimate free retinol in liver with absolute recovery 99.8% and 86.7 ± 3.6% respectively.

Using these three methods we could therefore estimate total vitamin A, retinol palmitate, retinol and by the difference the other retinol esters. These methods could be used in fat emulsion or liver. All of these analyses can be performed using one apparatus with different extraction techniques and different polarities of the eluant.

2.1.8 Other biochemical measurements

1) Retinol-binding protein (RBP)

RBP was measured on Radial Immunodiffusion (RID) plates (Behring-werke). Standard human serum (ORDT 02/03) (Behring-werke) was used with dilution. This method could detect RBP levels down to 5 mg/l. The normal range was 30-75 mg/l.

2) Prealbumin (PA)

PA was measured by RID in a similar way to RBP. This method could detect PA levels down to 30 mg/l. The normal range was 100-400 mg/l.

3) C-reactive protein (CRP)

C-RP was measured on the Abbott TDX Analyser by a fluorescence polarisation method. All reagents, standards and controls were supplied by Abbott laboratories. Minimum sample volume is 50 ul. The linearity of the method is from 0-250 mg/l. Higher concentrations required predilution in saline. The reference range is < 10 mg/l.

4) Transferrin

Transferrin was measured by a turbidimetric method on the Encore Centrifugal Analyser. The antiserum used was sheep Anti-transferrin supplied by the Scottish Antibody Production Unit (used at 1:40 dilution). The calibrator was calibrator 1 (human serum) supplied by Atlantic Antibodies. The sample was prediluted 1:25 in 4% polyethylene glycol (PEG) in saline and the calibrator was prediluted 1:40 in 4% PEG in saline and a standard curve was prepared to cover the range 0.25-5.0 g/l. Samples and standards were spun for 10 min at 3000 rpm. The pipettor 1000 was used to pipette prediluted sample or standard (10 µl), antiserum (250 µl) and diluent (50 µl) prior to analysis. Reference range 2.0-4.0 g/l.

5) Cholesterol

A kinetic colorimetric test (CHOD-PAP Method) (Boehringer Mannheim) was used to measure cholesterol on a centrifichem analyser (Baker).

6) Triglycerides (TG)

Triglyceride was measured by a fully-enzymatic colorimetric test (Merckotest). Triglycerides were hydrolyzed enzymatically to glycerol and free fatty acids by a special combination of lipases. The glycerol was measured enzymatically on a Centrifichem analyser and is proportional to the total triglyceride concentration.

7) Vitamin E

Plasma vitamin E was measured by a modification of the fluorometric method of Kahan (1966), and Thompson et al (1973). D-α-tocopherol was used as standard (concentration 25 umol/l) with excitation wavelength 300 nm, and emission wavelength of 326 nm.

3) RESULTS

3.1) GENERAL STUDIES ON SERUM RETINOL

3.1.1) Relation to time of day

The variation of serum retinol and retinol-binding protein were studied in 9 females, 10 males in relation to the time of collection at Glasgow Royal Infirmary. Volunteers aged 19-43 were studied, weight 48-83 kg and heights 159-185 cm. The blood samples were collected at 8 am, 12 noon, 2 pm, 5 pm and 10 pm. Samples correspond to after overnight fast; before and after lunch and dinner. The plasma was separated immediately and stored at -20°C until retinol and retinol-binding protein were analysed.

Table 30 shows that no significant difference in serum retinol levels have been found before and after ingestion of a normal meal (ie between 8 am and 12 noon, and 12 noon and 2 pm). However, there was a highly significant difference ($p < 0.001$) between paired 8 am and 10 pm samples (mean difference 0.23 µmol/l). The regression coefficient for 8 am and 10 pm samples was $r = 0.97$. Figure 20 shows the correlation between serum retinol and RBP at 5 pm ($r = 0.8$).

Discussion

It can be concluded that for individuals on a normal oral diet the time of collecting the blood specimen for retinol analysis must be carefully controlled. However, the effect of a meal on serum retinol can largely be ignored. Serum retinol levels measured by HPLC did not change after a normal or high oral dose of retinol palmitate (Section 3.2.3). These results also confirm that it is not necessary to collect specimens from

TABLE 30

SERUM RETINOL AT DIFFERENT TIMES DURING THE DAY
(MEAN ± 1 SD) n = 19

Time	8 am	12.00	2.00 pm	5.00 pm	10.00 pm
Mean µmol/l	3.1	3.1	3.0	3.0	2.9
SD	0.94	0.92	0.94	0.89	0.77
SE	0.22	0.21	0.22	0.21	0.18
%CV	30.9	29.6	30.9	29.9	26.8

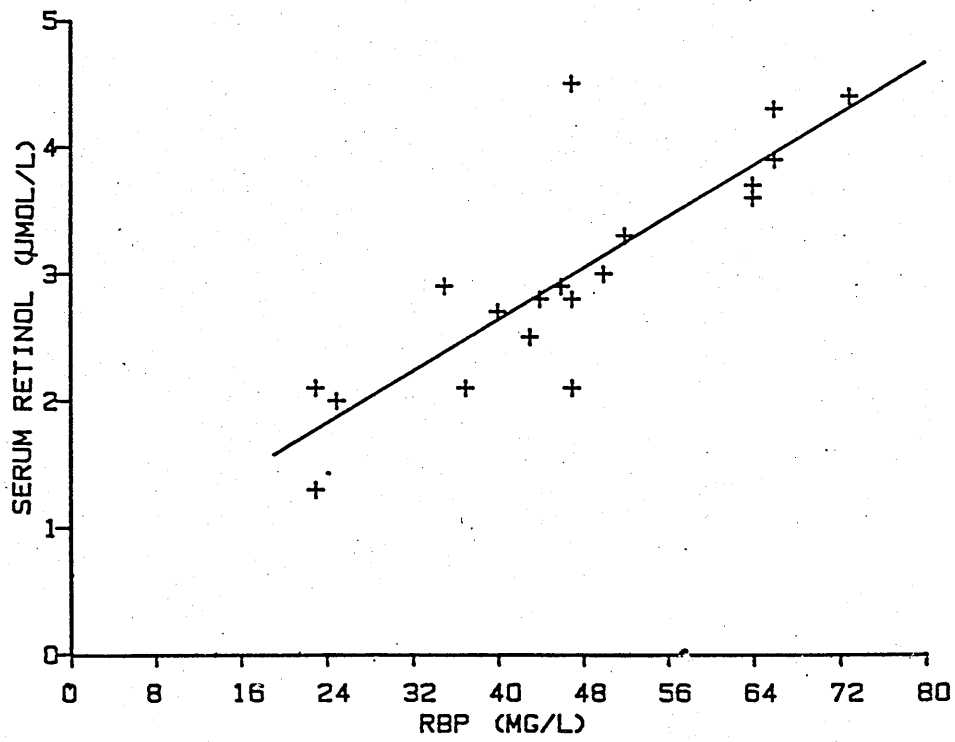


Figure 20: Correlation between serum retinol and retinol-binding protein in volunteers

patients in the fasting state. In all future studies every effort was therefore taken to obtain specimens from patients in the morning.

3.1.2) Normal Range

A random sample of 70 persons with no known ill health and between the age range 40-86 born in the Eastern District of Glasgow were studied (Section 3.3.1.1). A fasting sample of blood was taken from them and serum retinol measured (Table 31).

From this it was concluded that the reference range for males is approximately 1.2 - 5.6 µmol/l (mean ± 2 SD) and 1.3-4.5 µmol/l in females.

3.1.3) Stability of serum retinol

Venous blood samples were obtained from 3 healthy laboratory staff members, who had been previously found to have low, medium and high serum retinol concentration. The serum was kept at -20°C for up to 3 months. Each week the samples were thawed and serum retinol was measured by HPLC. Table 32 shows the precision of 30 analyses of each pool over the 3 month period and there was no change in concentration from the first week (0.4, 1.4, 3.8) to last week (0.4, 1.3, 3.7).

In the past the stability of retinol in frozen serum or plasma samples has been assessed primarily in samples stored in the dark for less than a year. The results obtained are dependent on the methods used for analysis (Table 33).

On the other hand, other factors may affect the stability eg temperature of storage, the volume of the sample since the larger the volume, the better is vitamin A stability Frolik and Olson (1984), handling and storage condition, and the

TABLE 31

SERUM RETINOL FOR NORMAL SUBJECTS (mean ± SD)

Subjects	No. of subjects	Serum retinol (μmol/l)
Total	70	3.2 ± 0.9
Male	27	3.4 ± 1.1
Female	31	2.9 ± 0.8

TABLE 32

STABILITY OF SERUM RETINOL (µmol/l)

Concentration	First week	Last week	Mean	CV %
Low	0.4	0.4	0.4	7.0
Medium	1.4	1.3	1.3	3.4
High	3.8	3.7	3.8	4.1

TABLE 33

STABILITY OF SERUM RETINOL IN PREVIOUS STUDIES

Author	Method used	Temperature (°C)	Storage period	Comment
Bessey et al 1946	UV	-20	4.5 months	no change
Kahan, 1966	Fluorescence	-20	several weeks	no change
Parkinson and Gal (1972)	Carr-Price	-20	1 week	higher
Bieri et al, 1979	reverse phase HPLC with UV	-20	3.5-8.5 months	20% decrease (J G Bieri, personal communication)

freezing and thawing procedure. Kark et al (1981) reported that up to 12 cycles of freezing and thawing did not influence plasma retinol as measured by trifluoroacetic acid method (Neeld and Pearson, 1963). In our study (Figure 21) we did not find any change during a 3 month period of repeated freezing and thawing and hence further samples were stored under these conditions.

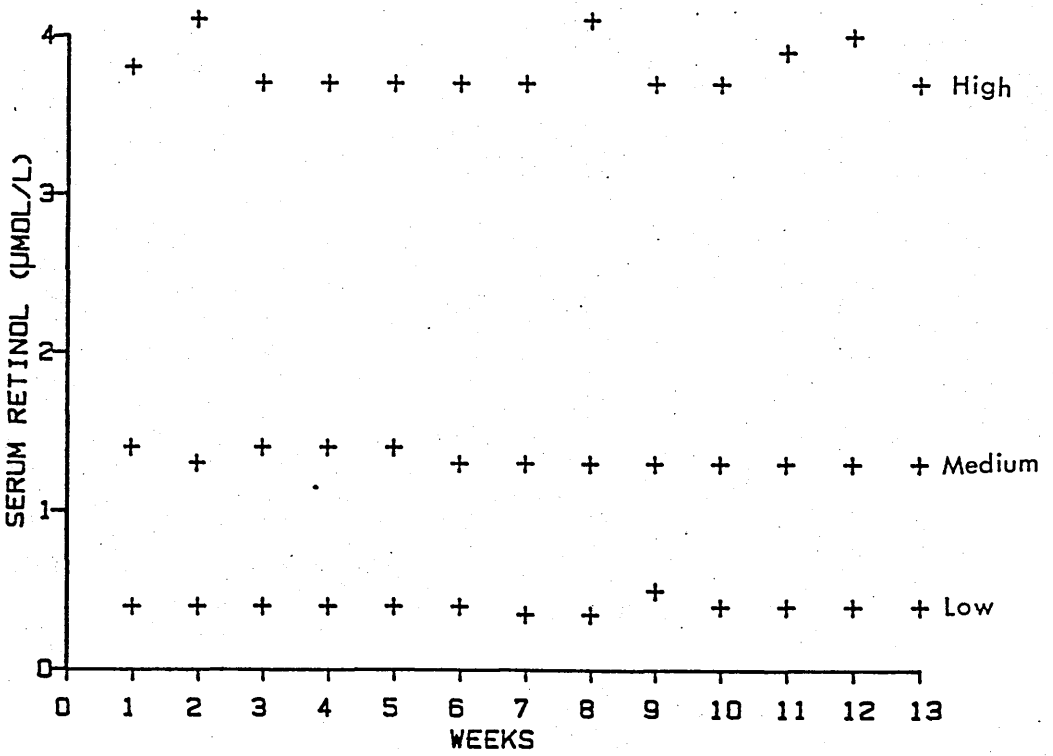


Figure 21: Stability of serum retinol in 3 human pools stored for 3 month periods.

3.2) NUTRITIONAL STUDIES

3.2.1) Stability of retinol palmitate in Intralipid solution (20%)

Vitlipid^{R} (10 ml) containing retinol palmitate (750 µg/10 ml) was added to 500 ml Intralipid (20%) under aseptic conditions. Care was taken during preparation to protect the contents of the Intralipid bottle from exposure to daylight. An administration set (Transcoden Lensahn, W Germany) with 15 µm filter and air inlet was connected and adjusted to deliver 50 ml/hour. The bottle was hung from a stand, 1 metre above ground level. The bottle was exposed to daylight, 0.45 metre from north facing windows. The experiments were started at 9 am and were organised in the period September and October. Samples were collected at 2 hour intervals into glass bottles. The last sample was allowed to stand in daylight and was further sampled at intervals to 168 hours.

Separate bottles of Intralipid 20% emulsion containing a similar amount of retinol palmitate were prepared. One was covered with a black plastic bag and stored at room temperature, and the other was kept in the dark at 4°C. They were sampled at similar times to the above. Solutions were analysed for retinol palmitate by the optimized HPLC method described previously (Section 2.1.5.2). 1 g of each mixture was dissolved in 10 ml isopropanol. The experiment (3 bottles/experiment) was repeated 4 times.

Results

A typical result of the retinol palmitate content in Intralipid 20% emulsion exposed to daylight is shown in Figure 22. At 24 hour, 40% of retinol palmitate had been lost, whereas by 168 hours only 20%

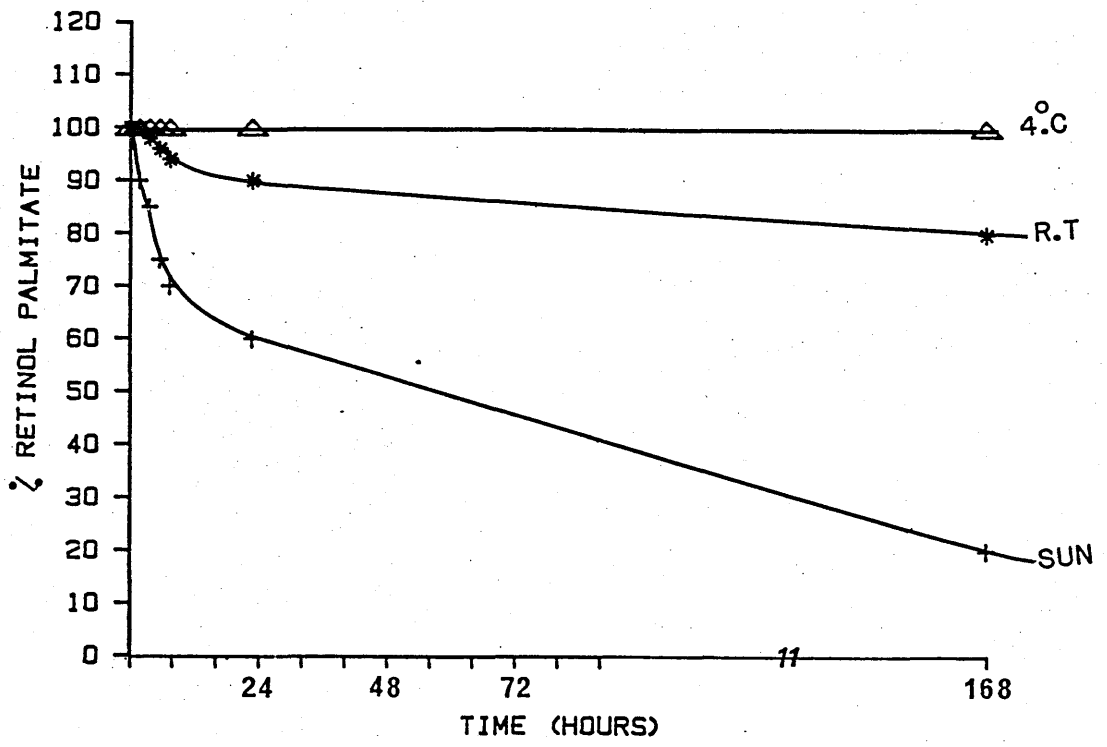


Figure 22: Stability of retinol palmitate in Intralipid emulsion for one week at 4°C (Δ), room temperature (*) or in sunlight (+).

remained. When it was stored at room temperature and protected from daylight, or stored at 4°C, there was no significant loss of retinol palmitate after 24 hours. Storage in the dark at room temperature for one week led to loss of almost 20%. Repetition of the study gave virtually identical results.

Discussion

The stability and delivery of retinol palmitate in these emulsions was studied for one week using the HPLC procedure. It was found that after 24 hour of exposure to daylight 60% of retinol palmitate was still present.

Allwood (1982) using 3 L bags found only 7% retinol palmitate was delivered, assuming that infusion (100 ml/hour) was exposed to 12 hours daylight, followed by 12 hours darkness, with distance of 0.3metres from windows. In his study, with the same conditions as above except that a shade was put in the window, only 51% of retinol palmitate was delivered. Rates of degradation would be influenced by the flow rate which determines the exposure time. Other factors also may affect degradation, eg time of day, direction of the light, weather condition, location of the hospital, the amount of daylight exposure in the ward and the time of year. Our experiment was carried out in September and October, while Allwood carried his out in May and July.

He estimated retinol palmitate by adding Multibionta (BDH) solution to 3 L bags (1000 µg/l), while we estimated retinol palmitate by adding Vitlipid^{R} (Kabi Vitrum) to 500 ml Intralipid 20% emulsion with concentration 1500 µg/l. Both studies confirmed that an emulsion stored at room temperature and protected from daylight, or at 4°C did not lose any significant retinol palmitate over a 24 hour period.

Riggle and Brandt (1986) found that the decrease of available retinol in parenteral nutrition solutions was not due to photodecomposition. The decrease was due to uptake by the tubing, which was dependent on flow rate. A mean decrease of 26 to 67% was seen if flow rate was changed from 75 ml/hour to 10 ml/hour. In their study they used retinol, whereas recently Gutcher et al (1984) have suggested using the retinol palmitate form of vitamin A as a means of circumventing these losses. However, Kishi et al (1981) also using retinol palmitate demonstrated 50% loss of vitamins, which was controlled partially by protecting the bag from light. In this study we used retinol palmitate and the loss is undetectable if stored at room temperature or 4°C for 24 hours.

3.2.2) Clearance of IV retinol palmitate from venous blood

Three patients receiving IVN, had the Intralipid 20% infusion, containing Vitlipid, commenced at 1 am and completed at 9 am. Blood samples were collected at 9 am, 11 am and 1 pm. Serum retinol and retinol palmitate were measured by HPLC, while total vitamin A was measured by fluorimetric method. Triglyceride was measured as well in these three patients. Table 34 shows the results.

Discussion

Table 34 shows that retinol palmitate given intravenously is cleared from the blood sample after about 2 hours from the end of the infusion. Serum retinol levels did not change during infusion, although the total vitamin A shows a change at 9 am. At the same time serum triglyceride shows a high value at 9 am. It can be concluded that for measurement of serum retinol

TABLE 34

SERUM RETINOL PALMITATE, RETINOL, TOTAL VITAMIN A AND TRIGLYCERIDE AT DIFFERENT TIMES AFTER INFUSION

No.	Time	Retinol Palmitate (μmol/1)	Retinol (μmol/1)	Total Vitamin A (μmol/1)	Triglyceride (mmol/1)
1	9 am	0.25	1.4	1.8	4.35
	11 am	0.1	1.3	1.3	1.85
	1 pm	0.04	1.3	1.1	1.05
2	9 am	0.14	0.3	0.9	3.15
	11 am	0.04	0.4	0.5	1.05
	1 pm	0.00	0.4	0.4	0.60
3	9 am	0.49	1.7	3.5	13.25
	11 am	0.14	1.7	2.0	3.05
	1 pm	0.06	2.1	1.7	0.75

levels by the fluorometric method in patients treated with intravenous infusion, samples should be taken 4 hours after infusion of fat emulsion, so that there is minimal interference due to triglyceride or retinol palmitate.

3.2.3) Clearance of orally ingested retinol palmitate

In this study 6 normal male subjects age range between 28-63 arrived on a Sunday evening to GRI and were fasted until next morning. Blood samples were taken from the subjects early in the morning then a meal was given consisting of:

380 ml full cream

20 ml granulated sugar

20 ml dried milk

40 ml flavourite syrup

300,000 units of retinol palmitate (Crusha), equal to 160 mg/l = 558.4 µmol/l.

The formula diet was made to one pint with distilled water, which was drunk within 15 minutes. Two hours after the meal, blood samples were taken and then after each two hours until 8 hours, or 10 hours. Normal hospital food was taken at 4 hours and 8 hours and a further blood sample was taken after 24 hours.Serum retinol and retinol palmitate were measured by HPLC and total retinoids by fluorimetry, as well as triglyceride and retinol-binding protein (RBP).

Results

The retinol palmitate and retinol concentration in serum by HPLC as well as total retinoid concentration by fluorimetry and triglyceride concentration are illustrated in Figure 23-28 for each subject.

Figure 23: The change in serum (1) total retinol (violet) by fluorometry; (2) retinol (black); (3) Retinol palmitate (green) by HPLC and (4) Triglyceride (red) after an oral dose of 300,000 IU of retinol palmitate in a fatty meal.

Subject 1

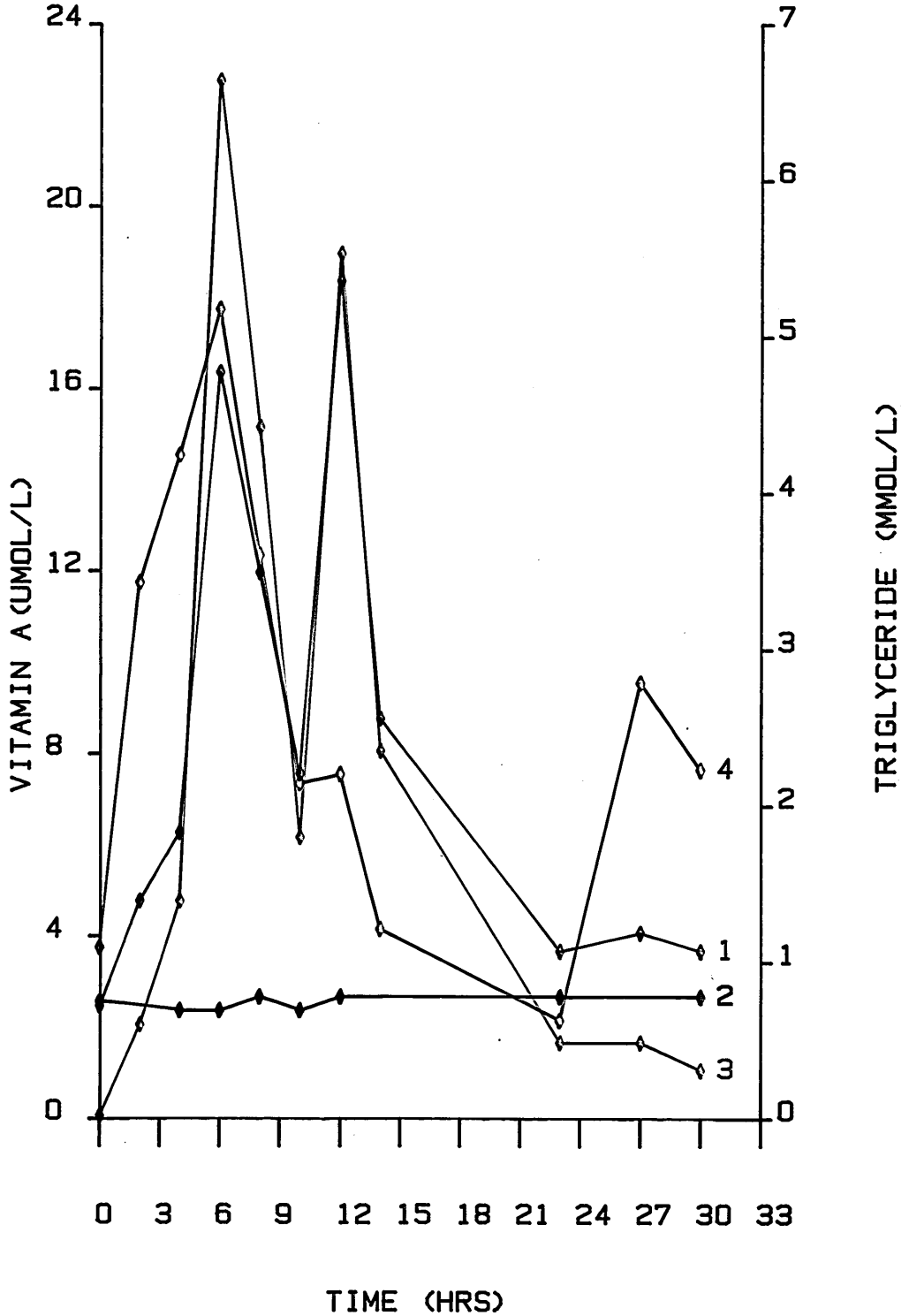

24
20
16
12
8
4
0
VITAMIN A (UMOL/L)
7
6
5
4
3
2
1
0
TRIGLYCERIDE (MMOL/L)
0 3 6 9 12 15 18 21 24 27 30 33
TIME (HRS)
1
2
3
4

Figure 24: The change in serum (1) total retinol (violet) by fluorometry; (2) retinol (black), (3) Retinol palmitate (green) by HPLC and (4) Triglyceride (red) after an oral dose of 300,000 IU of retinol palmitate in a fatty meal.

Subject 2

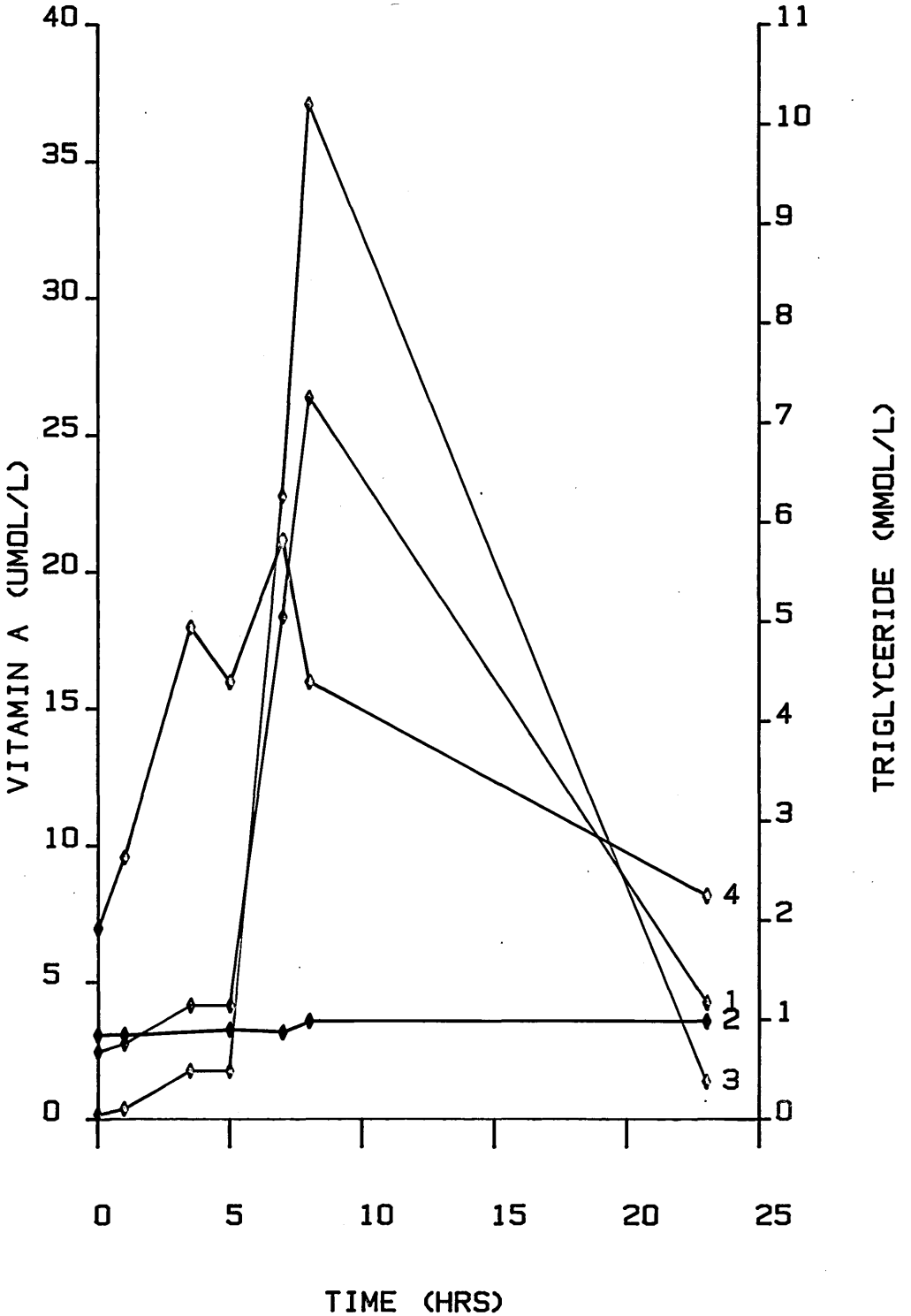
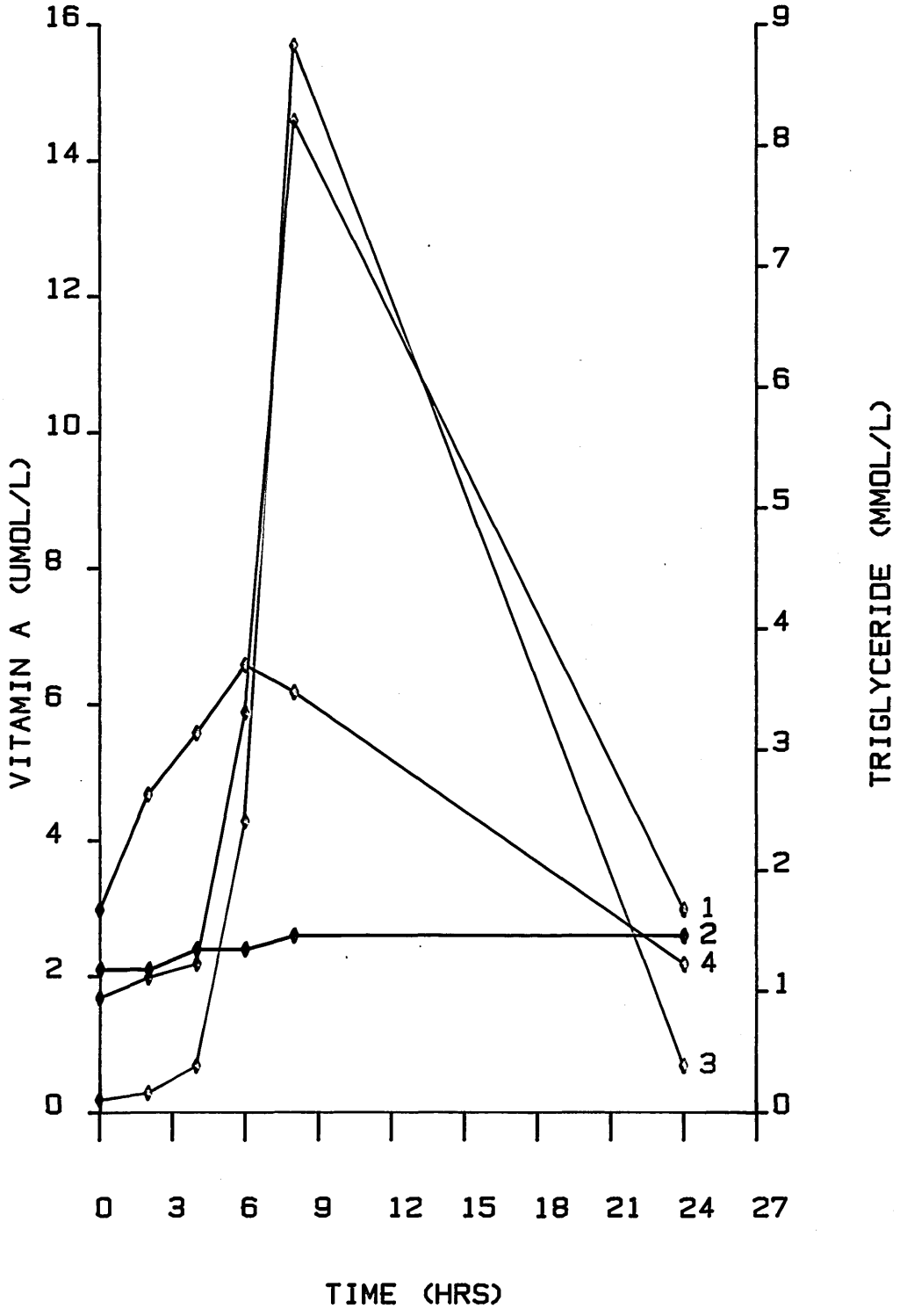

VITAMIN A (UMOL/L)
TRIGLYCERIDE (MMOL/L)
TIME (HRS)
40
35
30
25
20
15
10
5
0
11
10
9
8
7
6
5
4
3
2
1
0
0
5
10
15
20
25
1
2
3
4

Figure 25: The change in serum (1) total retinol (violet) by fluorometry; (2) retinol (black); (3) retinol palmitate (green) by HPLC and (4) triglyceride (red) after an oral dose of 300,000 IU of retinol palmitate in a fatty meal.

Subject 3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14
12
10
8
6
4
2
0
VITAMIN A (UMOL/L)
9
8
7
6
5
4
3
2
1
0
TRIGLYCERIDE (MMOL/L)
1
2
4
3
0
3
6
9
12
15
18
21
24
27
TIME (HRS)

Figure 26: The change in serum (1) total retinol (violet) by fluorometry; (2) retinol (black); (3) retinol palmitate (green) by HPLC and (4) triglyceride (red) after an oral dose of 300,000 IU of retinol palmitate in a fatty meal.

Subject 4

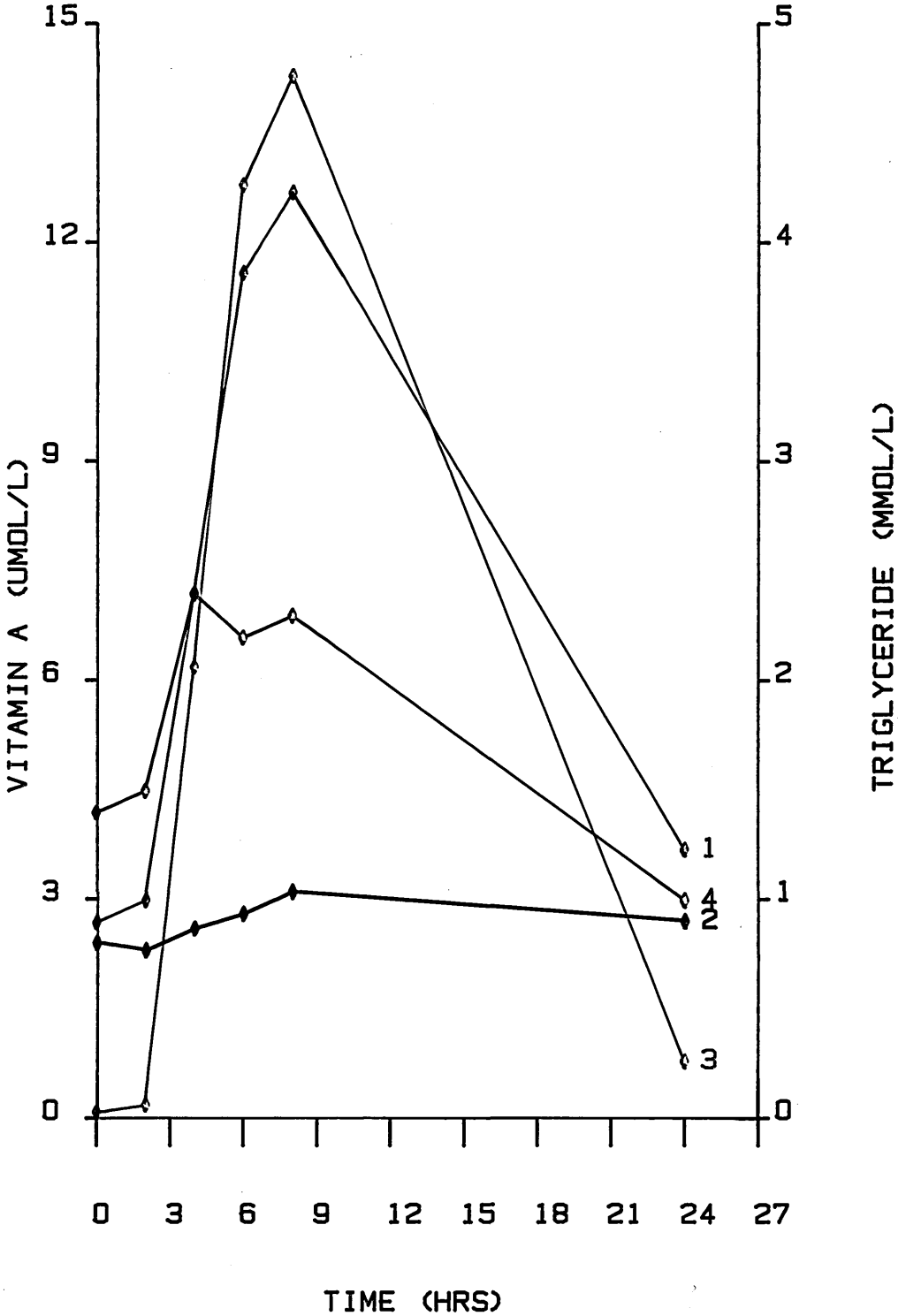

15
12
9
6
3
0
5
4
3
2
1
0
VITAMIN A (UMOL/L)
TRIGLYCERIDE (MMOL/L)
1
4
2
3
0 3 6 9 12 15 18 21 24 27
TIME (HRS)

Figure 27: The change in serum (1) total retinol (violet) by fluorometry; (2) retinol (black); (3) retinol palmitate (green) by HPLC and (4) triglyceride (red) after an oral dose of 300,000 IU of retinol palmitate in a fatty meal.

Subject 5

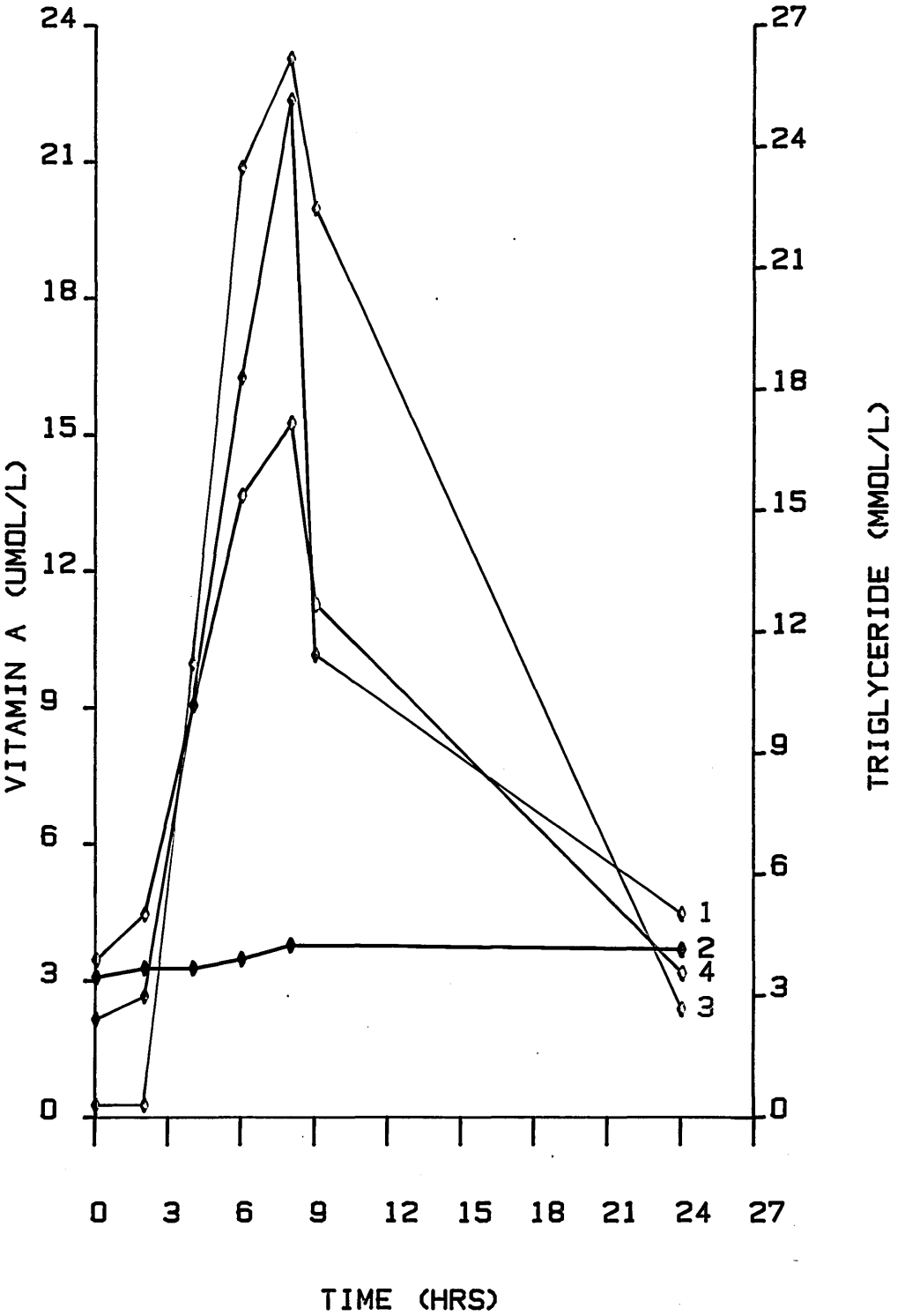
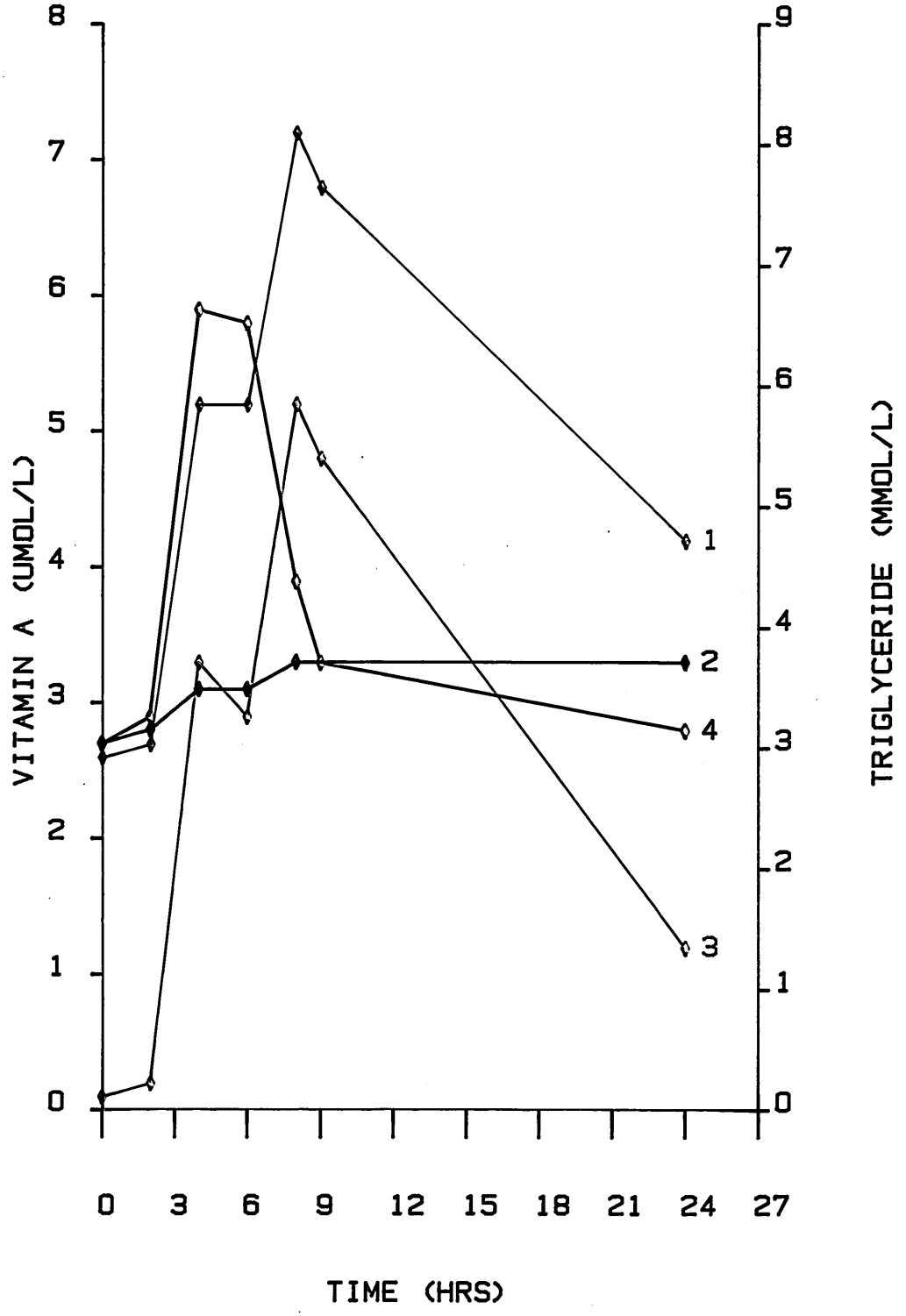

24
21
18
15
12
9
6
3
0
VITAMIN A (UMOL/L)
27
24
21
18
15
12
9
6
3
0
TRIGLYCERIDE (MMOL/L)
1
2
4
3
0 3 6 9 12 15 18 21 24 27
TIME (HRS)

Figure 28: The change in serum (1) total retinol (violet) by fluorometry; (2) retinol (black); (3) retinol palmitate (green) by HPLC and (4) triglyceride (red) after an oral dose of 300,000 IU of retinol palmitate in a fatty meal

Subject 6


VITAMIN A (UMOL/L)
TRIGLYCERIDE (MMOL/L)
TIME (HRS)
0
1
2
3
4
5
6
7
8
9
0
3
6
9
12
15
18
21
24
27
1
2
3
4

It can be seen that retinol palmitate increased in each subject with the peak concentration reached by 8 hours. In one subject in whom further samples were taken there was a second peak of retinol palmitate after 12 hours.

Serum retinol levels measured by HPLC remained unchanged following a dose of retinol palmitate in all subjects. The total serum retinoids measured by fluorimetry followed that of retinol palmitate peaks for each subject except that the concentration was lower than expected from the HPLC results.

The figures also show that triglyceride increased rapidly and reached a peak after 6 hours. It then dropped sharply at 8 hours and reached the starting concentration in all subjects by 24 hours.

Discussion

Vitamin A is a lipid soluble micronutrient. The factors affecting its transport and absorption in the small intestine have been summarised in Sections 1.3 and 1.4.

In lymph, Vitamin A appears as retinyl ester, while in plasma over 90% of vitamin A occurs as retinol. Immediately after a dose of retinol palmitate and when chylomicron floods the circulation, there is a substantial increase in the retinyl ester (Goodman et al 1965). The ratio of ester:alcohol is rapidly restored to fasting levels as the tissues, particularly the liver, take up the newly absorbed vitamin (Underwood, 1974). Thus for a short time, which was found to be approximately 8 hours, retinyl esters bound to lipoprotein enter the blood stream via the lymph appearing in plasma. Then they find their way into tissues where they are stored. On the other hand, absorbed

triglyceride in the lymph also appears in chylomicrons (which are very small particles composed of a core of triglyceride, with an outer film of phospholipid, cholesterol and protein). In some subjects, retinol palmitate and cholesterol began to increase in serum after triglyceride. Chylomicron catabolism is normally a rapid process that proceeds in at least two phases; one is to activate enzyme lipoprotein lipase, leading to the hydrolysis of chylomicron core triglycerides. This phase of chylomicron metabolism takes place at the surface of capillary endothelium of extrahepatic tissues. The hydrolysis of triglyceride results in shrinkage of the chylomicron core and transfer of redundant surface components into HDL (Goodman and Blaner, 1984). The long process of chylomicron metabolism leads to the formation of a smaller spherical lipoprotein particle called a chylomicron remnant (Redgrave, 1970). The remnant is depleted in triglyceride and more enriched in cholesteryl ester, phospholipid and protein. Hazz and Bierman (1976) found that these remnants are also relatively enriched in vitamin A, which may explain why triglyceride falls before vitamin A. This may also be due to quicker absorption, hydrolysis and re-esterification of triglyceride than vitamin A.

Serum retinol and retinyl esters are transported by separate carrier proteins. (Ganguly et al 1952). RBP shows no change after an ordinary meal or high oral dose of retinol palmitate, because it correlated with retinol which did not change during the study (HPLC) over the 24 hour period.

It is particularly interesting to us that the serum retinol did not change, despite the large increase in serum retinol palmitate. This is presumably due to the rapid uptake and storage of retinyl esters by the liver in the post absorptive period.

Mejia et al (1984) indicated that up to 4 hours after a vitamin A-rich meal, there was no significant difference in serum concentration of retinol, RBP and carotenoids. Although retinol palmitate was not measured in his study, our results agreed with this finding. It is possible that after ingesting a meal, the rate of gastric emptying was not fast enough to provide at one point an amount of retinol for intestinal absorption, sufficient to cause a detectable change in the serum concentration of the vitamin.

Kasper et al (1979) found in an experiment carried out on female subjects that total vitamin A concentration reached a peak 3 to 7 hours after oral administration of 300,000 IU (160 mg/l) of retinol palmitate. The time depended on the type of fibre added to the diet formula. Dietary fibre appears to increase the rate of absorption of retinol palmitate. When no fibre was added, a similar rate of absorption was found as in our study.

Retinol and its esters are the only naturally-occurring retinoids that fluoresce appreciably under normal conditions. Goodman and Leslie (1972) suggested that when retinol is attached to specific proteins in plasma such as retinol-binding protein (RBP) the fluorescence is much enhanced. Because retinol palmitate does not bind to RBP there is no increase in

fluorescence. Moreover, retinol acetate which is used for standardisation of the fluoresence assay, may fluoresce better than retinol palmitate. This may explain why serum levels of retinol palmitate measured by HPLC are higher than serum levels of total vitamin A measured by the fluorimetric method.

3.2.4) Studies on patients

3.2.4.1) Home TPN (IVN)

A number of patients require IVN at home as a result of major intestinal resection or severe inflammatory disease leading to malabsorption. A register of such patients in the United Kingdom is kept at the Department of Surgery, Hope Hospital, Salford. As part of a review of the micronutrient status of these patients samples were requested from the consultants in charge of patients in various parts of the country. These patients are an interesting group to study, not only because they have been maintained on artificial nutrition for prolonged periods, but also because they are not acutely ill, the effect of any acute phase reaction on measurements should be small. We therefore measured plasma retinol, retinol-binding protein, albumin and C-reactive protein (CRP) on 20 of these patients, and related this to the vitamin A intake and duration of nutritional support. These patients were supplied with IVN from local hospital pharmacies or a commercial company (Travenol). Some patients had a small amount of oral intake. Intravenous fat soluble vitamins were given eg 'Multibionta' infusion 5900 ug of retinol palmitate, and was provided daily for most patients, or as Vitlipid Adult infusion 750 µg ranging from daily - twice per week. Table 35 shows the amount given (µg/day) for each patient.

TABLE 35

RETINOL PALMITATE GIVEN (µg/day) TO HOME TPN PATIENTS

Patient No.	Retinol Palmitate (µg/day)	Patient No.	Retinol Palmitate (µg/day)	Patient No.	Retinol Palmitate (µg/day)
1	3107	9	Oral	17	750
2	750	10	3000	18	214
3	750	11	214	19	2140
4	750	12	oral	20	3214
5	oral	13	214	21	3000
6	3214	14	3000	22	214
7	oral	15	3000	23	-
8	3000	16	214	24	107

Result

The mean plasma level for retinol (3.4 ± 2.2 µmol/l) was not significantly different from a normal population (3.2 ± 0.9 µmol/l) (Figure 29). Five patients had high levels between 5.9 - 8.5 µmol/l; six patients showed low levels between 0.3 -1.6 µmol/l. Figure 30 shows the lack of correlation (r = 0.2) between serum retinol levels and the amount of vitamin A which had been taken (µg/day).

RBP and albumin were measured as an assessment of protein status to show the relation to vitamin A status. Figure 31 shows the correlation between RBP and serum retinol (r = 0.98). The mean level of albumin was 33.5 ± 7.0 g/l and there was a poor correlation (r = 0.2) with RBP (Figure 32).

C-reactive protein (CRP) was measured and there was no correlation between CRP and serum retinol or RBP (r = 0.1 and r = 0.01) respectively, while there was a negative correlation between CRP and albumin (r = 0.6). These results are inconclusive because only one patient had a significantly elevated CRP (> 50 mg/l). This patient had a low serum retinol, RBP and albumin.

Discussion

The relationship between retinol and RBP is discussed in Section 1.4. Patients with high levels of serum retinol show also high RBP levels, although two of these patients received as little as 214 µg/day and 750 µg/day intravenous vitamin A. There was a good correlation between serum retinol and RBP (r = 0.98). There was no correlation with albumin. Figure 30 shows a poor correlation between vitamin A intake/day and plasma retinol. This

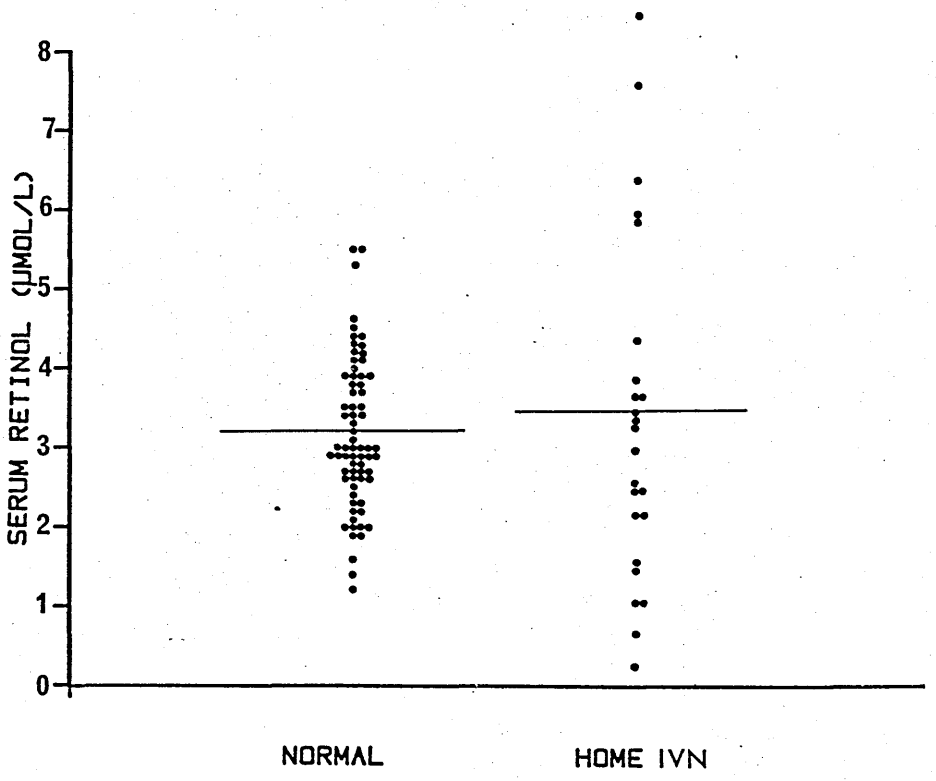


Figure 29: Serum retinol in normal individuals and patients on home IVN

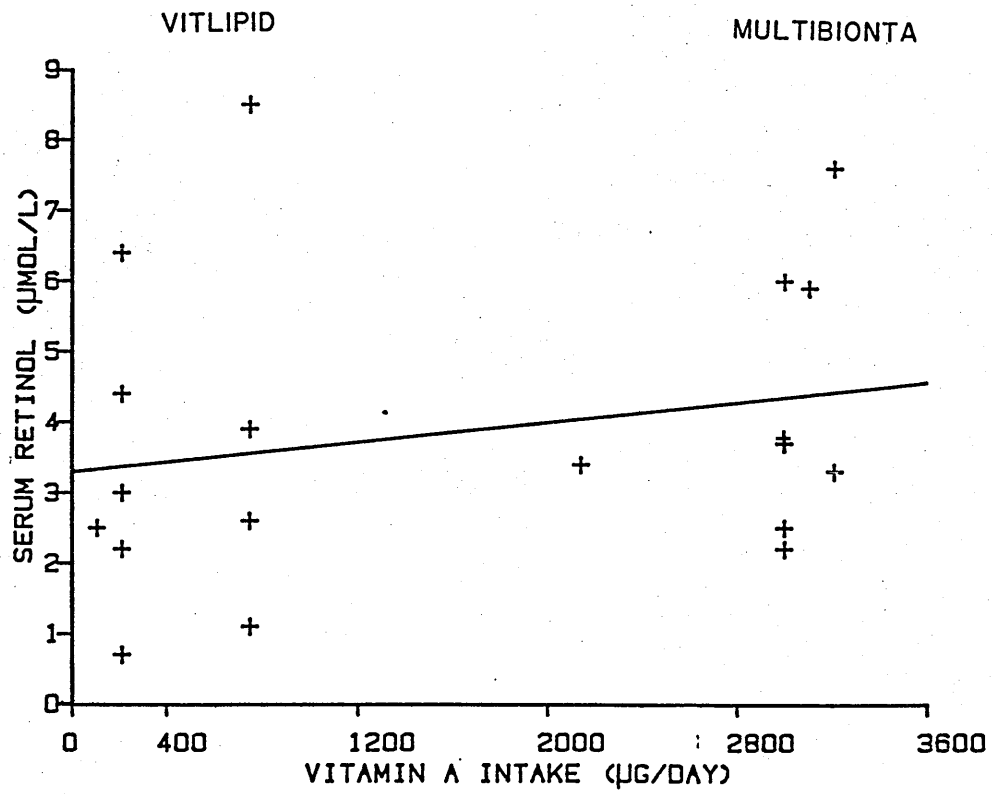


Figure 30: Correlation between vitamin A intake and serum retinol in home IVN patients

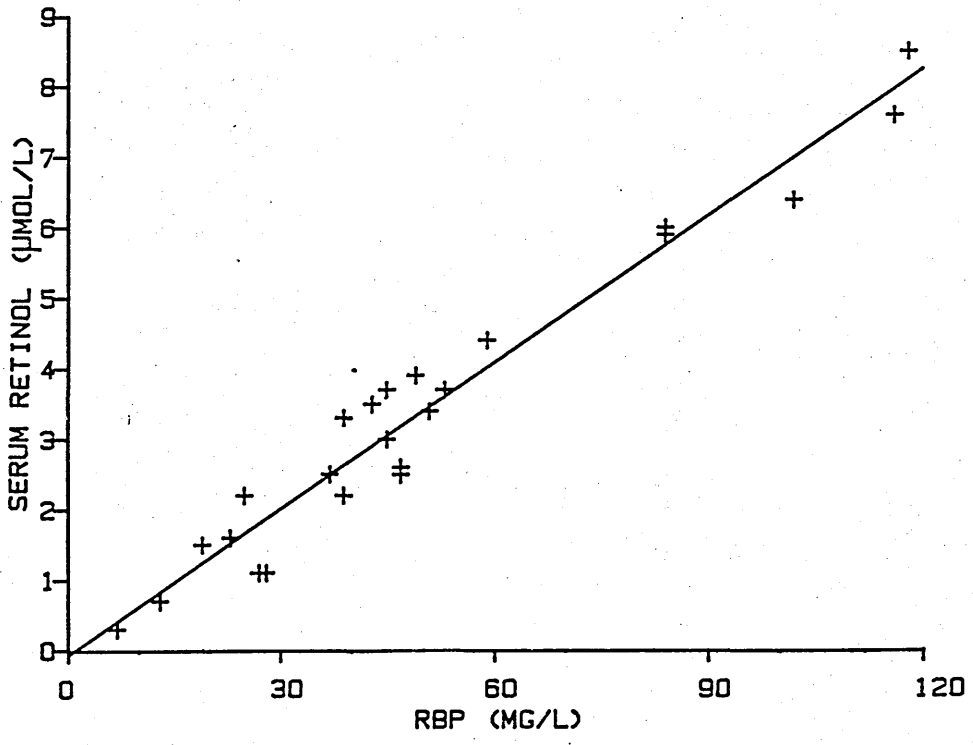


Figure 31: Correlation between serum retinol and retinol-binding protein in home IVN patients.

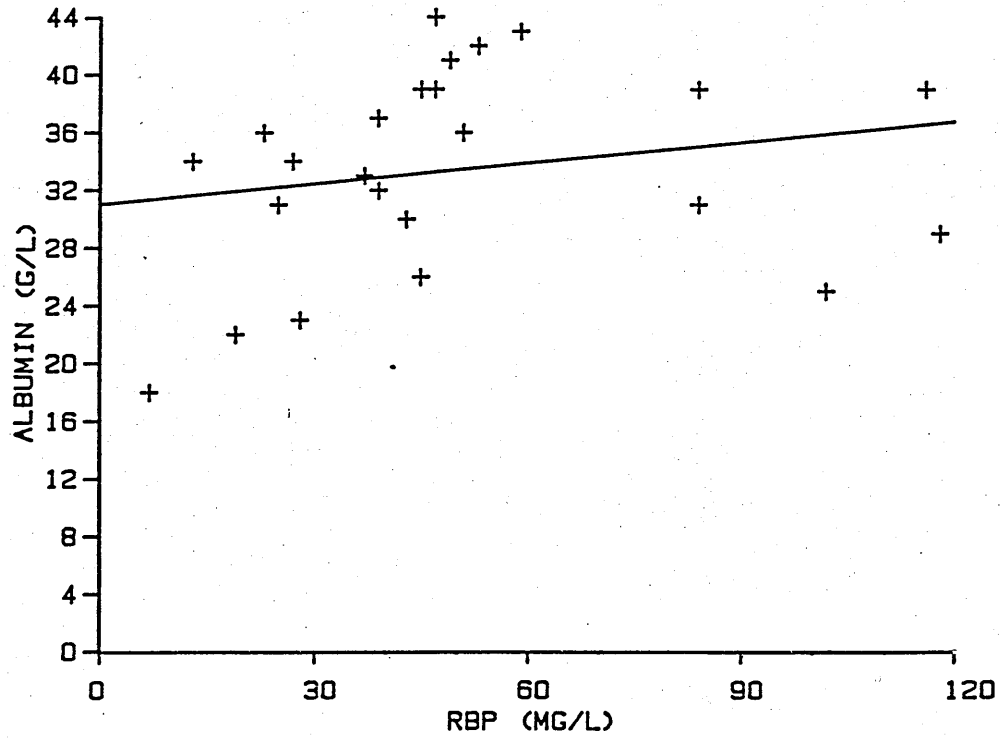


Figure 32: Correlation between albumin and retinol-binding protein in home IVN patients.

is presumably because vitamin A is stored in the liver, so the amount which has been taken should not be the same as that released from the liver. Four of the six patients who had low levels of plasma retinol were not receiving intravenous vitamin A supplements, and two were receiving only 214 and 750 µg/day intravenously. These patients also had low serum RBP, albumin concentration, two having concentrations of RBP and albumin (7,13 mg/l and 18,34 g/l) respectively. Perhaps, the plasma retinol was a reflection of protein depletion rather than retinol deficiency, or this may due to net protein catabolism in response to infection.

During the acute phase response, which is observed within hours or days after the onset of infection or inflammation, the liver dramatically increases the synthesis of certain proteins, whereas albumin synthesis is reduced (Dinarello, 1984). One of the proteins increased is C-reactive protein, which begins to rise very rapidly soon after injury (Kushner, 1982). Pepys and Baltz, (1983) found that CRP can rise from <10 to 200 mg/l in inflammation. Approximately 50% of the home IVN patients had a normal CRP (<10) implying no inflammation and the rest were slightly higher with the range 11-71, but only one had a high concentration indicating acute inflammation.

The lack of correlation between CRP and RBP/retinol or albumin reflects the small range of CRP concentration. Therefore retinol concentrations are probably not affected by an acute phase reaction in these patients, and these concentrations therefore indicate protein nutritional status as well as vitamin status. Most of these patients studied had normal serum albumin,

suggesting good protein status, although other markers of protein status, eg skeletal muscle may be helpful. Serum retinol in these patients was therefore probably a reasonable marker of vitamin A status.

The low serum retinol in some patients may also be due to inadequate supplement of vitamin A orally, leading to poor absorption. Ten of the patients, 4 oral and 6 IV, had been given less than the vitamin A daily intake recommended by HMSO (UK) 1979 (750 µg) or RDA by NA of Sciences (USA) 1980, (1000 µg) and 50% of these patients had levels of serum retinol below the normal range. It is interesting how many patients received high intakes of vitamin A in comparison with the RDA. Although there was no correlation of plasma retinol with level of input, none of the patients with an intake of > 2000 µg/day had a low serum retinol concentration. As far as we know none of these patients had any clinical evidence of vitamin A toxicity. Further studies on liver biopsies would be necessary to clarify the adequacy of vitamin A status in these patients.

3.2.4.2) Determination of serum retinol in surgical patients receiving nutritional support

Ten surgical patients, 4 male and 6 female, mean age 47 years, range 17-72, received nutritional support before and after operation. Five patients were fed intravenously according to a standard regimen, throughout treatment. The average daily intake was 3000 ml containing 14 gm N and 3000 kcal (2000 kcal carbohydrate/1000 kcal fat). The infusion was provided with all water soluble nutrients (except vitamins) in a 3 L bag over 24 hours, together with a separate infusion of Intralipid 20% (500 ml) containing 1 vial Solivito^{R} (water-soluble vitamins), and 1 vial Vitlipid^{R} (fat-soluble vitamins) which was given over 8 hours. 2500 IU (750 µg) retinol palmitate were provided each day. Four patients received enteral nutrition orally, with different amounts of tube feed. One patient received a combination of IVN and enteral nutrition at different times.

The duration of the study was 2 months - 3 years. Nutritional assessment included regular (every 1-2 weeks) measurement of transferrin, albumin, RBP, serum retinol and body weight. CRP was measured for 7 patients only (Figures 33-42; Tables 36 and 37).

Discussion

In plasma, retinol is transported bound to RBP in a one to one molar relationship. RBP is a serum protein with a short biological half-life and serum levels of retinol appear intimately associated with the capacity of the liver to synthesis RBP and hence mobilise vitamin A stored in the liver (Ingenbleek

TABLE 36: DIAGNOSIS AND COMMENTS ON SURGICAL PATIENTS

Patient No.	Age	Sex	Diagnosis	Type of Feeding	Pre or post operation	Mean N intake (g)	Mean intake of retinol palmitate (IU)	Operation	Comment
1	60	M	Cancer - oesophagus	IVN	pre	12.3	3300	Endoscopy	Body weight began to rise slowly after IVN given. Albumin and transferrin did not show any consistent change. RBP and retinol in serum increased together and dropped when the CRP rose (due to infection) and the N intake was decreased to 9 gN. It began to rise again when 14 gN were provided and the CRP fell (Figure 33)
2	46	F	Large bowel polyps	IVN (long term)	pre & post	11.0	2500	Division of small bowel re-anastamosis of ileostomy & mucus fistula	Pre-operation period shows no change in all proteins and serum retinol. During the early post-operation period all proteins, serum retinol and body weight decreased, whereas CRP increased. During the prolonged period post-operation there was an increase in all except CRP which dropped to low levels. RBP and serum retinol fell, during a period when N intake was maintained, but retinol palmitate was not continued. CRP increase also correlated with fall in retinol/RBP conc. When CRP fell, the vitamin supplements were re-introduced retinol/RBP concentration increased (Figure 34)
3	17	F	Pancreatic abscess & fistula	IVN	post	13.3	2500	Resection head of pancreas Reanastamosis of stomach to duodenum Drainage of pancreatic abscess	There was a marked increase in all measurements over the 6 week period post-operation except for albumin and CRP. Albumin showed a decrease after operation and increase 13 days later, while CRP showed a sharp decrease and reached normal range (< 10 mg/l) after 18 days, with a good inverse correlation of r = 0.9 with RBP. In the post-operative period, serum retinol and RBP return to normal with IVN, and with the fall in CRP (Figure 35).
4	27	F	Crohn's Disease	IVN	pre	11.5	2500		All measurements increase during IVN and CRP remained within the normal range (< 10 mg/l). The increase in CRP after 4 weeks, IVN was not associated with a fall in proteins or retinol. At this time the IVN had been increased from 9-14 gN/day (Figure 36).
5	46	M	Zollinger Ellison Syndrome Pancreatic Fistula	IVN	post	10.7	3300	Distal pancreatectomy splenectomy Drainage 1 Drainage 2	RBP and serum retinol showed low level 12 days post-operatively which continued till day 53 post-operation. After the first drainage operation whilst on oral diet, they started to increase. Transferrin showed an increase, while body weight and albumin were unchanged. After the second drainage operation, body weight decreased, while all other measurements were increased. (Fig 37)

TABLE 37: DIAGNOSIS AND COMMENTS ON SURGICAL PATIENTS

Patient Number	Age	Sex	Diagnosis	Type of Feeding	Pre or post operation	Mean N intake (g)	Mean intake of retinol palmitate (IU)	Operation	Comments
6	72	M	Prostatic enlargement Chronic Pancreatitis	Gluten free diet	post	7.5	3566	Transurethral prostatectomy	All proteins, as well as serum retinol show an increase by 22 days post operation. Changes in CRP were mainly inverse of RBP and retinol. RBP and serum retinol did not return to normal, whilst CRP remained elevated (Figure 38)
7	46	F	Ca Larynx	Nasogastric clinifeed	post	8.9	2985	laryngectomy	There was a consistent increase in all measurements over the 3 week period following operation except CRP showed a decrease inverse to RBP with (r = 0.8), but remained higher than the normal range (Figure 39)
8		M	Ca Larynx	Nasogastric	pre & post	12.4	4153	laryngectomy	All measurements showed marked decrease after operation, except CRP which showed an increase with correlation of r = -0.62 with RBP. After 24 days, CRP had returned to within the normal range (Figure 40)
9	62	F		Jejunostomy	post	8.5	2860	laparotomy & insertion of feeding jejunostomy	There was an early increase in all measurements post operation. Thereafter, she developed diarrhoea and the serum retinol, RBP and other proteins decreased. Vials of extra vitamin A supplement (Ketovite) and Vitamin C were provided, and all measurements subsequently increased (Figure 41)
10	47	F	Chronic Pancreatitis Prolonged ileus	Nasogastric and IVN	Pre & post	9.8 16	3300 2500	choledochojejunostomy	During nasogastric feeding for 4 days preoperation, all measurements decreased except body weight. After operation when IVN was given, all measurements increased. On transfer to oral feeding, the RBP and serum retinol decreased. (Figure 42).

Figure 33: Patient 1. The change in serum:

1) RBP (black)
2) Albumin (violet)
3) Retinol (blue)
4) Transferrin (red)
5) Body weight (green)
6) CRP (brown),

prior to surgical operation

IVN = intravenous nutrition
Op = operation

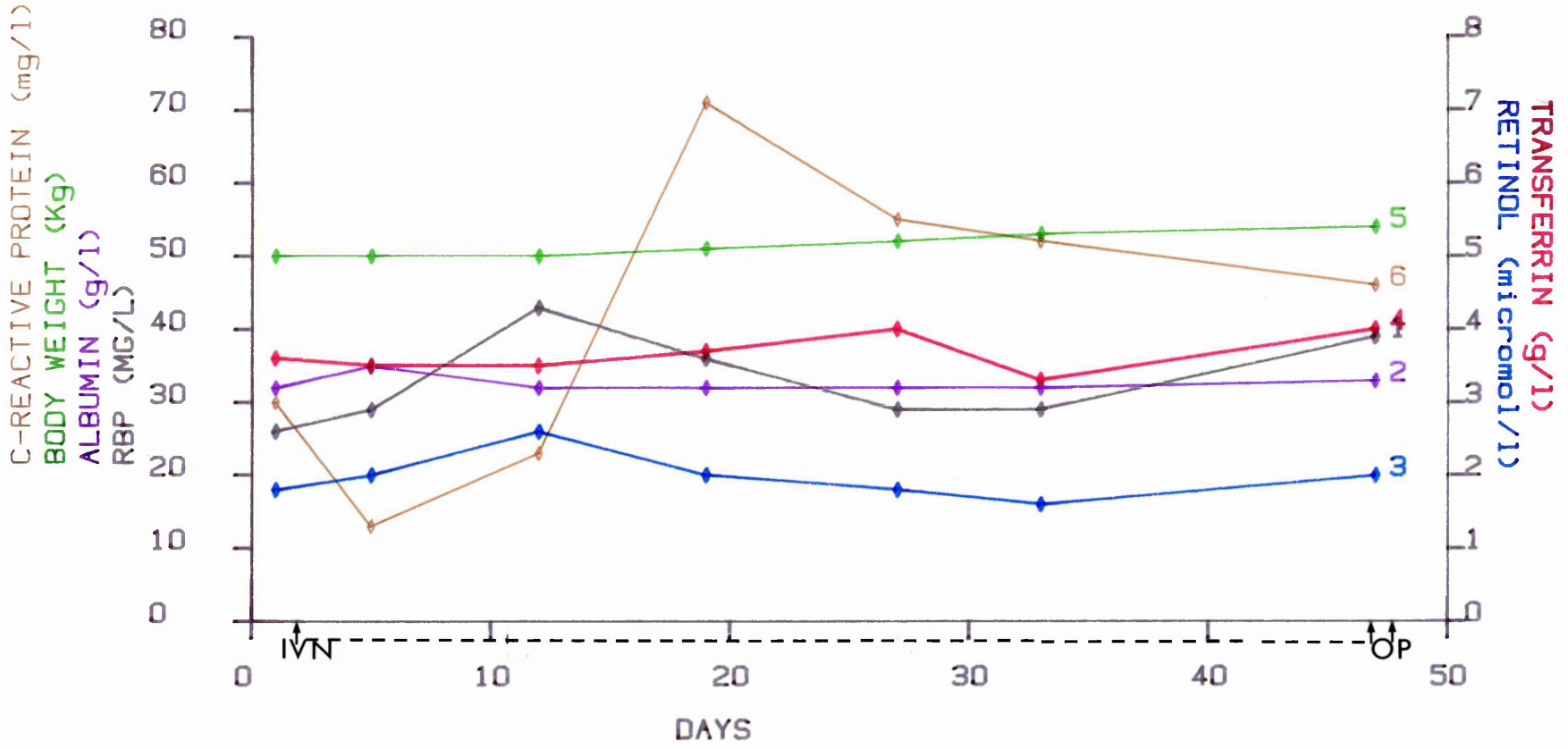

C-REACTIVE PROTEIN (mg/l)
BODY WEIGHT (Kg)
ALBUMIN (g/l)
RBP (MG/L)
TRANSFERRIN (g/l)
RETINOL (micromol/l)
80
70
60
50
40
30
20
10
0
8
7
6
5
4
3
2
1
0
IVN
OP
0
10
20
30
40
50
DAYS
5
6
4
1
2
3

Figure 34: Patient 2. The change in serum:

1) RBP (black)
2) Albumin (violet)
3) Retinol (blue)
4) Transferrin (red)
5) Body weight (green)
6) CRP (brown)

before and after surgical operation

IVN = intravenous nutrition
Op = operation

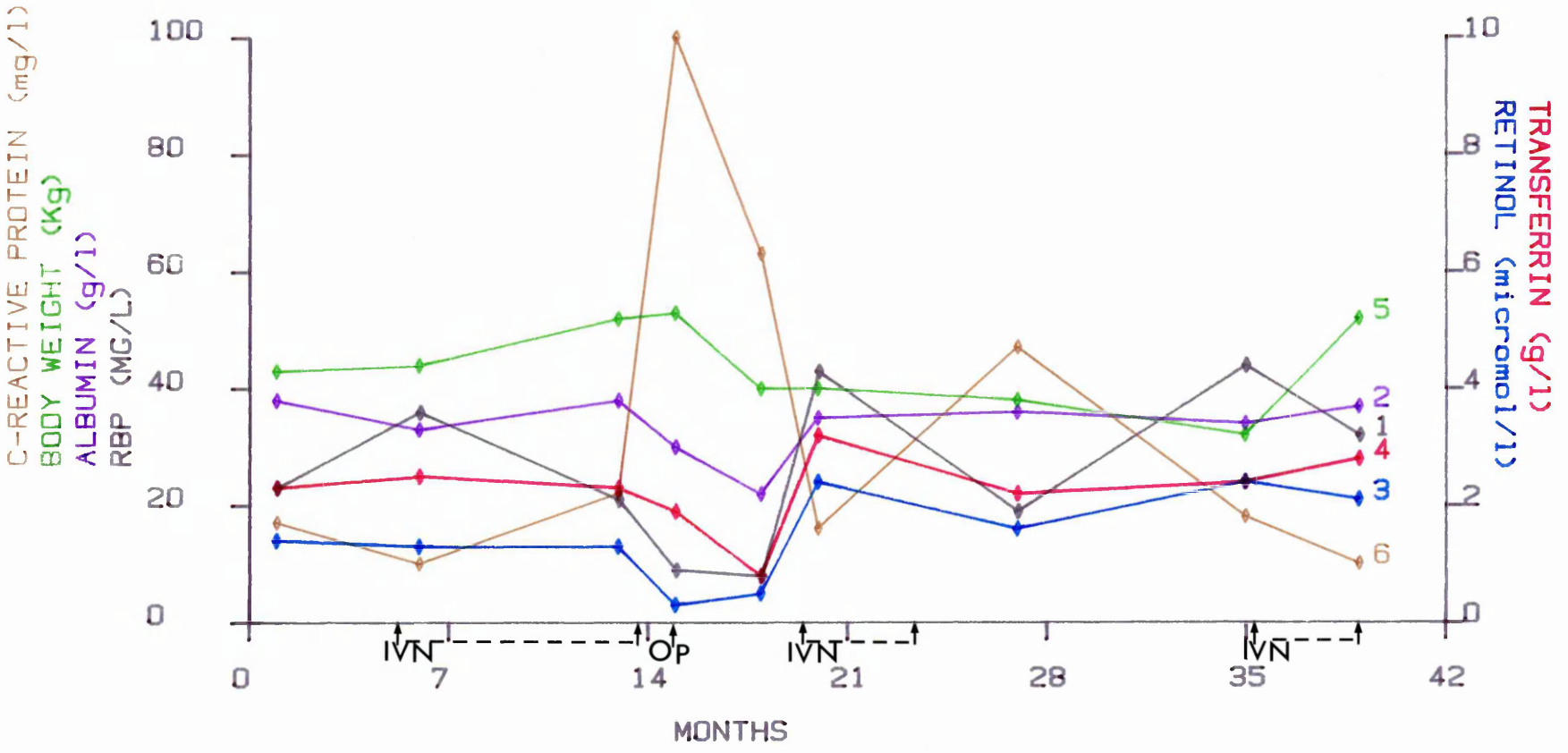
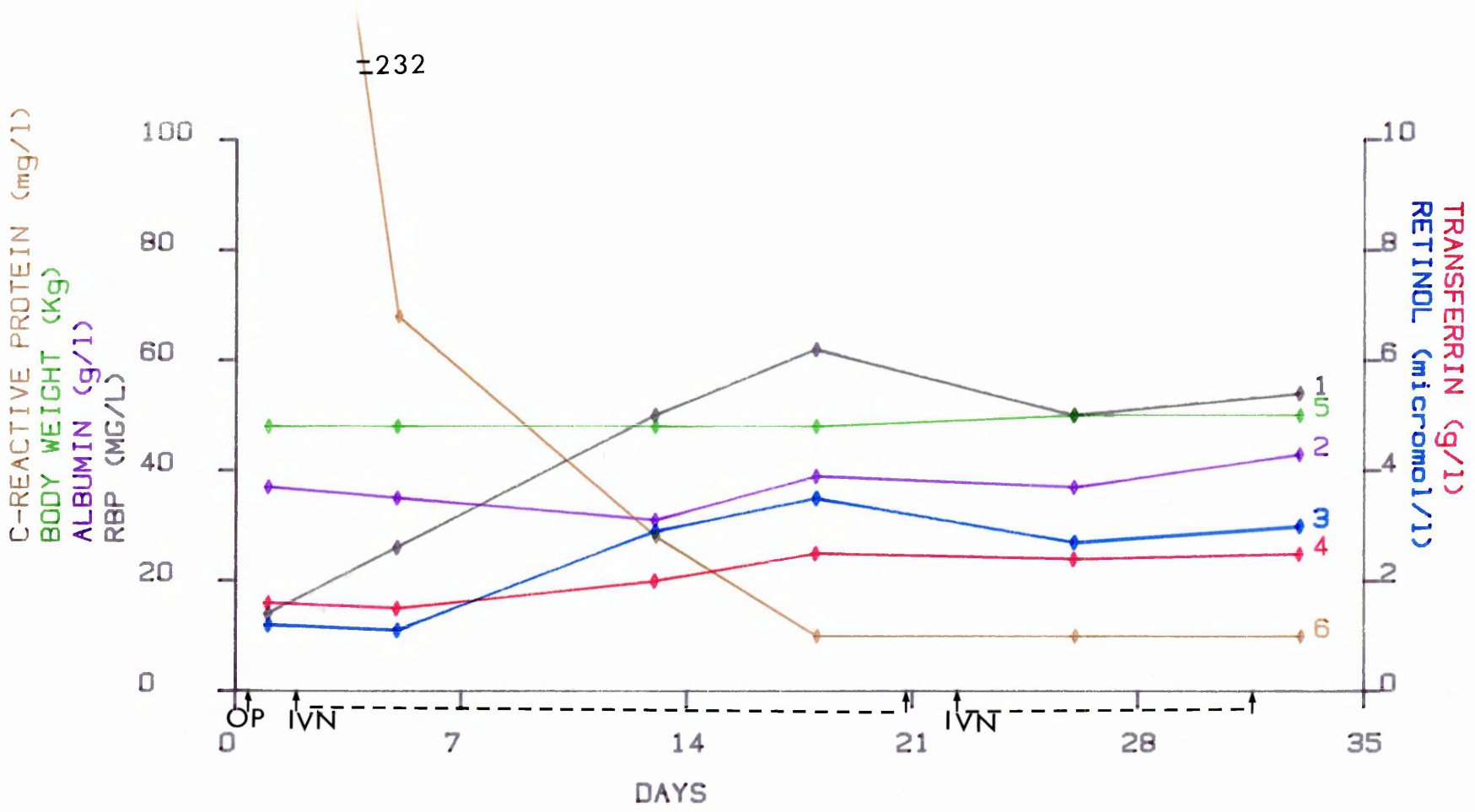

C-REACTIVE PROTEIN (mg/l)
BODY WEIGHT (Kg)
ALBUMIN (g/l)
RBP (MG/L)
TRANSFERRIN (g/l)
RETINOL (micromol/l)
100
80
60
40
20
0
10
8
6
4
2
0
5
2
1
4
3
6
IVN
OP
IVN
IVN
0
7
14
21
28
35
42
MONTHS

Figure 35: Patient 3. The change in serum:

1) RBP (black)
2) Albumin (violet)
3) Retinol (blue)
4) Transferrin (red)
5) Body weight (green)
6) CRP (brown)

after surgical operation

IVN = Intravenous nutrition
Op = operation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C-REACTIVE PROTEIN (mg/l)
BODY WEIGHT (Kg)
ALBUMIN (g/l)
RBP (MG/L)
TRANSFERRIN (g/l)
RETINOL (micromol/l)
100
80
60
40
20
0
10
8
6
4
2
0
1
5
2
3
4
6
OP
IVN
IVN
0
7
14
21
28
35
DAYS

Figure 36: Patient 4. The change in serum:

1) RBP (black)
2) Albumin (violet)
3) Retinol (blue)
4) Transferrin (red)
5) Body weight (green)
6) CRP (brown)

prior to surgical operation

IVN = Intravenous nutrition
Op = operation

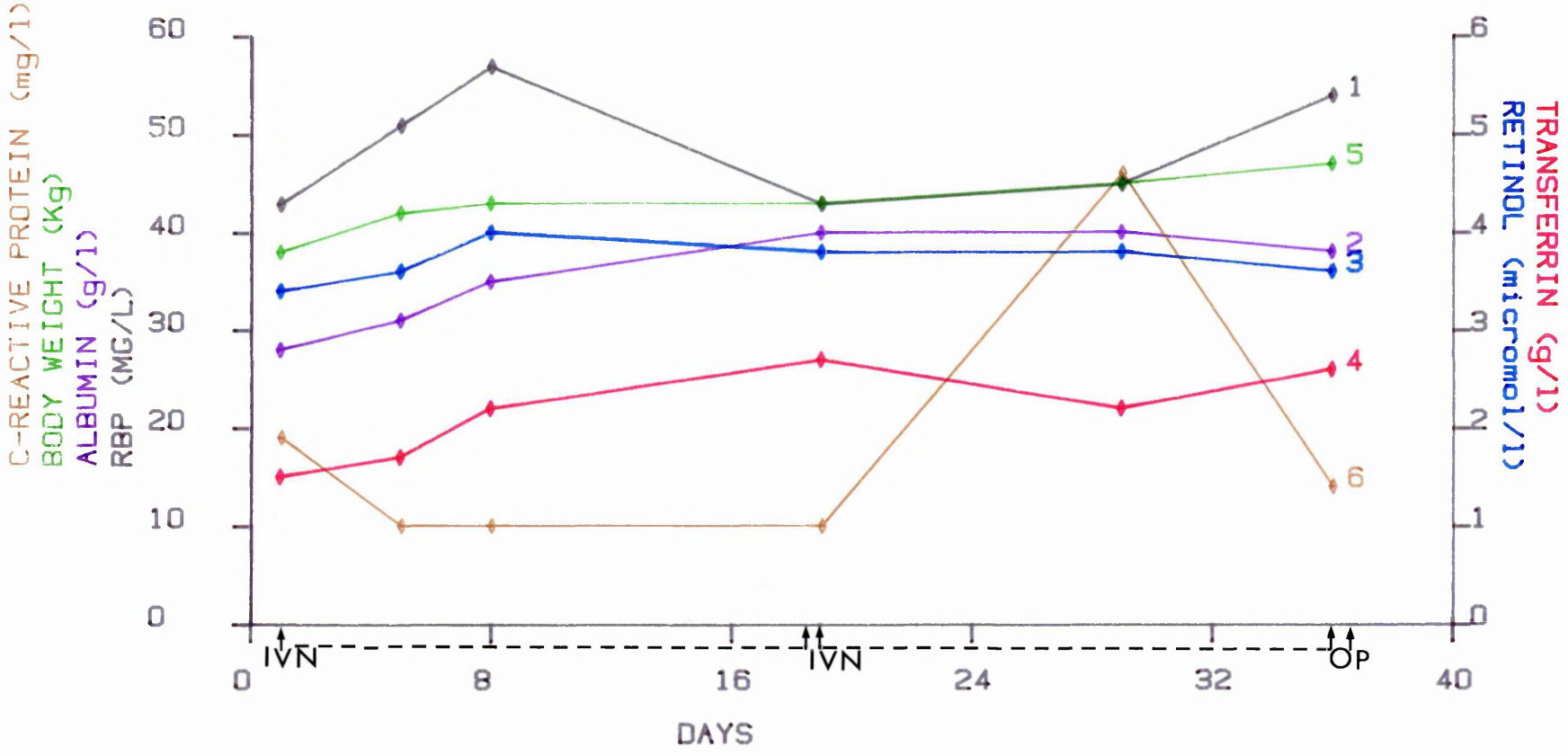

C-REACTIVE PROTEIN (mg/l)
BODY WEIGHT (Kg)
ALBUMIN (g/l)
RBP (MG/L)
TRANSFERRIN (g/l)
RETINOL (micromol/l)
60
50
40
30
20
10
0
6
5
4
3
2
1
0
1
5
2
3
4
6
IVN
IVN
OP
0
8
16
24
32
40
DAYS

Figure 37: Patient 5. The change in serum:

1) RBP (black)
2) Albumin (violet)
3) Retinol (blue)
4) Transferrin (red)
5) Body weight (green)

after surgical operation

Op = operation
IVN = intravenous nutrition
OD = oral diet
D = drainage

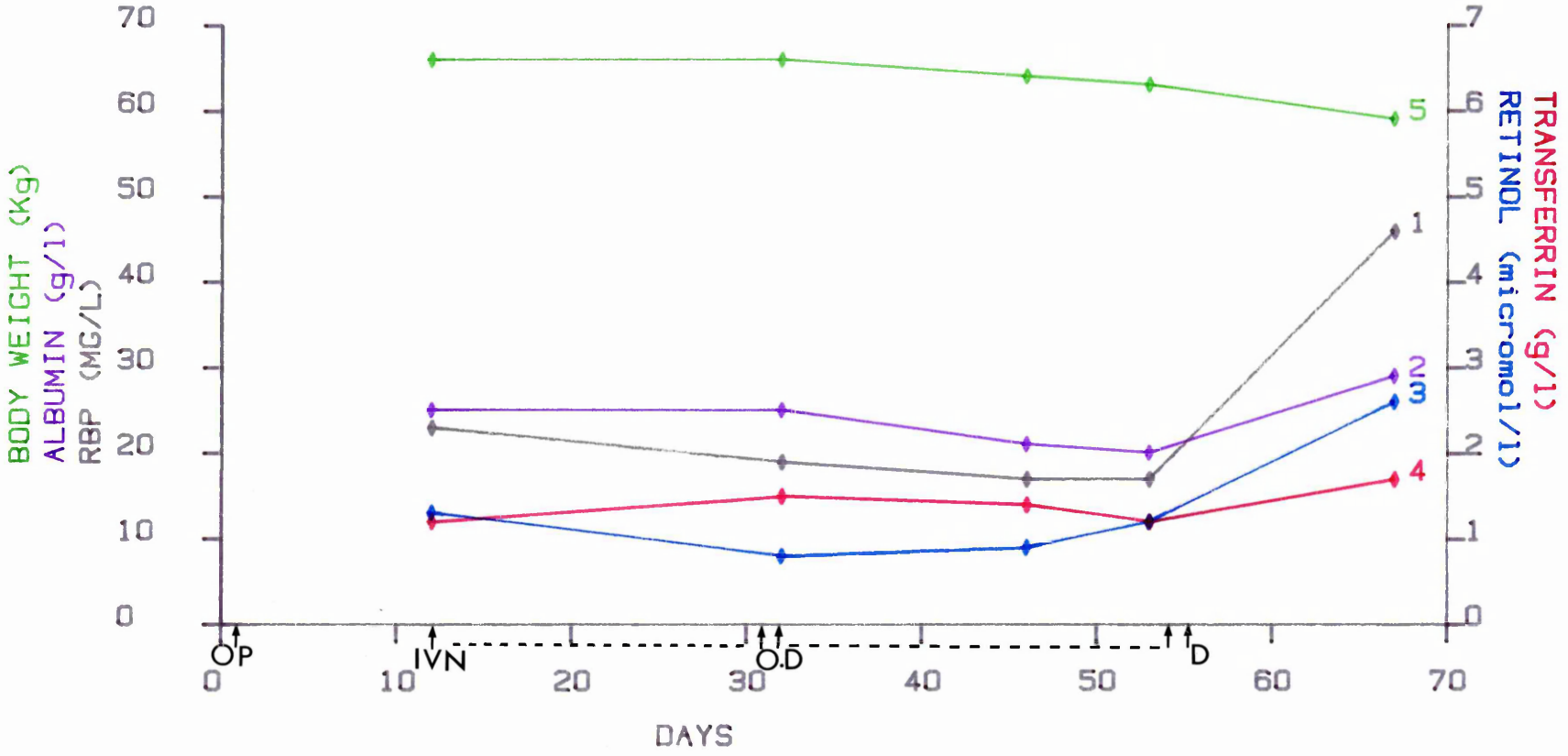

BODY WEIGHT (Kg)
ALBUMIN (g/l)
RBP (MG/L)
TRANSFERRIN (g/l)
RETINOL (micromol/l)
70
60
50
40
30
20
10
0
7
6
5
4
3
2
1
0
5
1
2
3
4
OP
IVN
O.D
D
0
10
20
30
40
50
60
70
DAYS

Figure 38: Patient 6. The change in serum:

1) RBP (black)
2) Albumin (violet)
3) Retinol (blue)
4) Transferrin (red)
5) Body weight (green)
6) CRP (brown)

after operation

Op = operation
GFD = gluten free diet
NG = naso-gastric feed

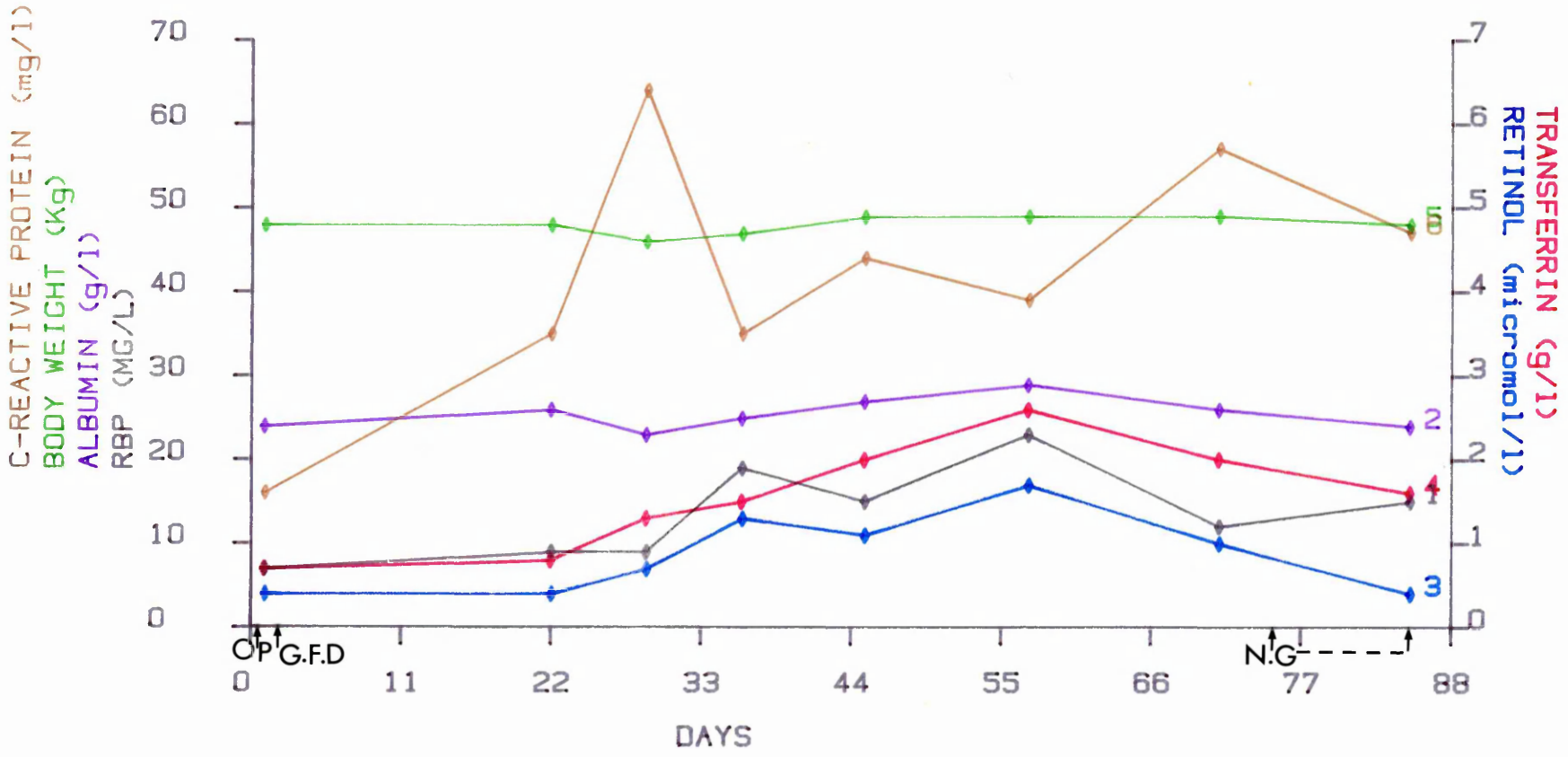

C-REACTIVE PROTEIN (mg/l)
BODY WEIGHT (Kg)
ALBUMIN (g/l)
RBP (MG/L)
TRANSFERRIN (g/l)
RETINOL (micromol/l)
70
60
50
40
30
20
10
0
7
6
5
4
3
2
1
0
5
2
4
1
3
O.P
G.F.D
N.G
0
11
22
33
44
55
66
77
88
DAYS

Figure 39: Patient 7. The change in serum:

1) RBP (black)
2) Albumin (violet)
3) Retinol (blue)
4) Transferrin (red)
5) Body weight (green)
6) CRP (brown)

after surgical operation

Op = operation
NG = nasogastric feed

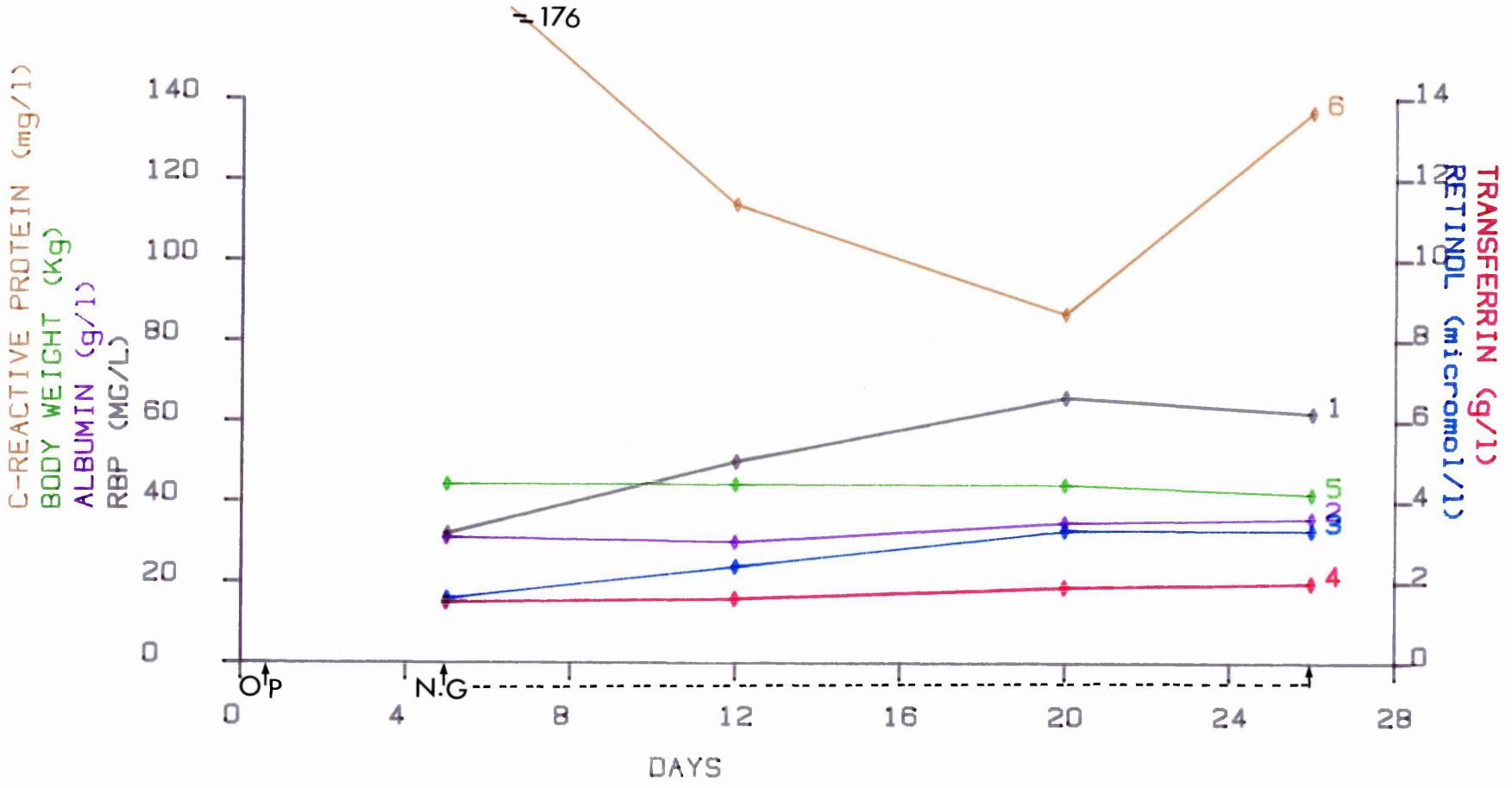

C-REACTIVE PROTEIN (mg/l)
BODY WEIGHT (Kg)
ALBUMIN (g/l)
RBP (MG/L)
TRANSFERRIN (g/l)
RETINOL (micromol/l)
176
140
120
100
80
60
40
20
0
14
12
10
8
6
4
2
0
6
1
5
2
3
4
O P
N.G
0
4
8
12
16
20
24
28
DAYS

Figure 40: Patient 8. The change in serum:

1) RBP (black)
2) Albumin (violet)
3) Retinol (blue)
4) Transferrin (red)
5) Body weight (green)
6) CRP (brown)

after surgical operation

Op = operation
NG = nasogastric feed

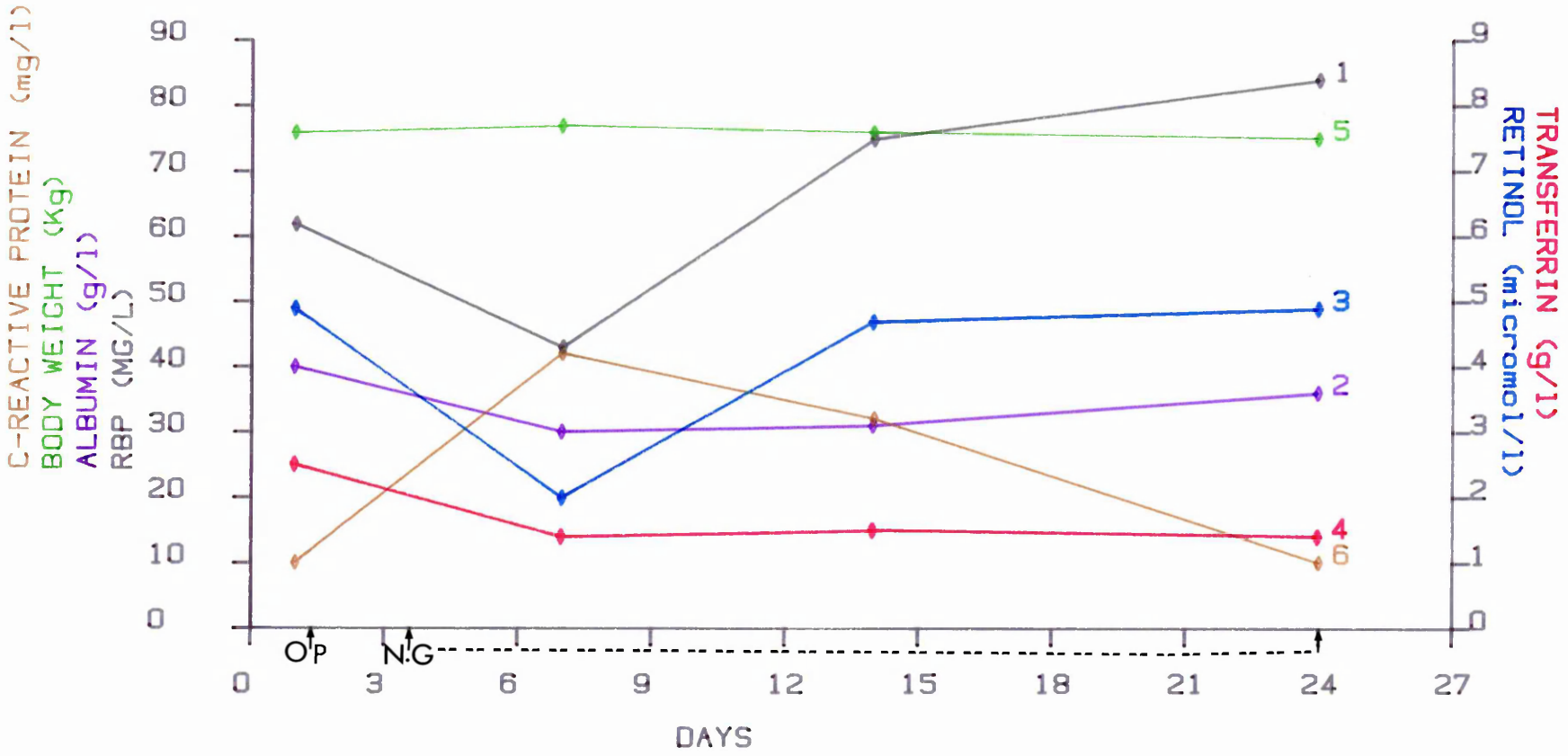

C-REACTIVE PROTEIN (mg/l)
BODY WEIGHT (Kg)
ALBUMIN (g/l)
RBP (MG/L)
TRANSFERRIN (g/l)
RETINOL (micromol/l)
90
80
70
60
50
40
30
20
10
0
9
8
7
6
5
4
3
2
1
0
O P
N G
0
3
6
9
12
15
18
21
24
27
DAYS
1
5
3
2
4
6

Figure 41: Patient 9: The change in serum:

1) RBP (black)
2) Albumin (violet)
3) Retinol (blue)
4) Transferrin (red)
5) Body weight (green)

after surgical operation

Op = Operation
JF = Jejunostomy feed
EV = Extra vitamin

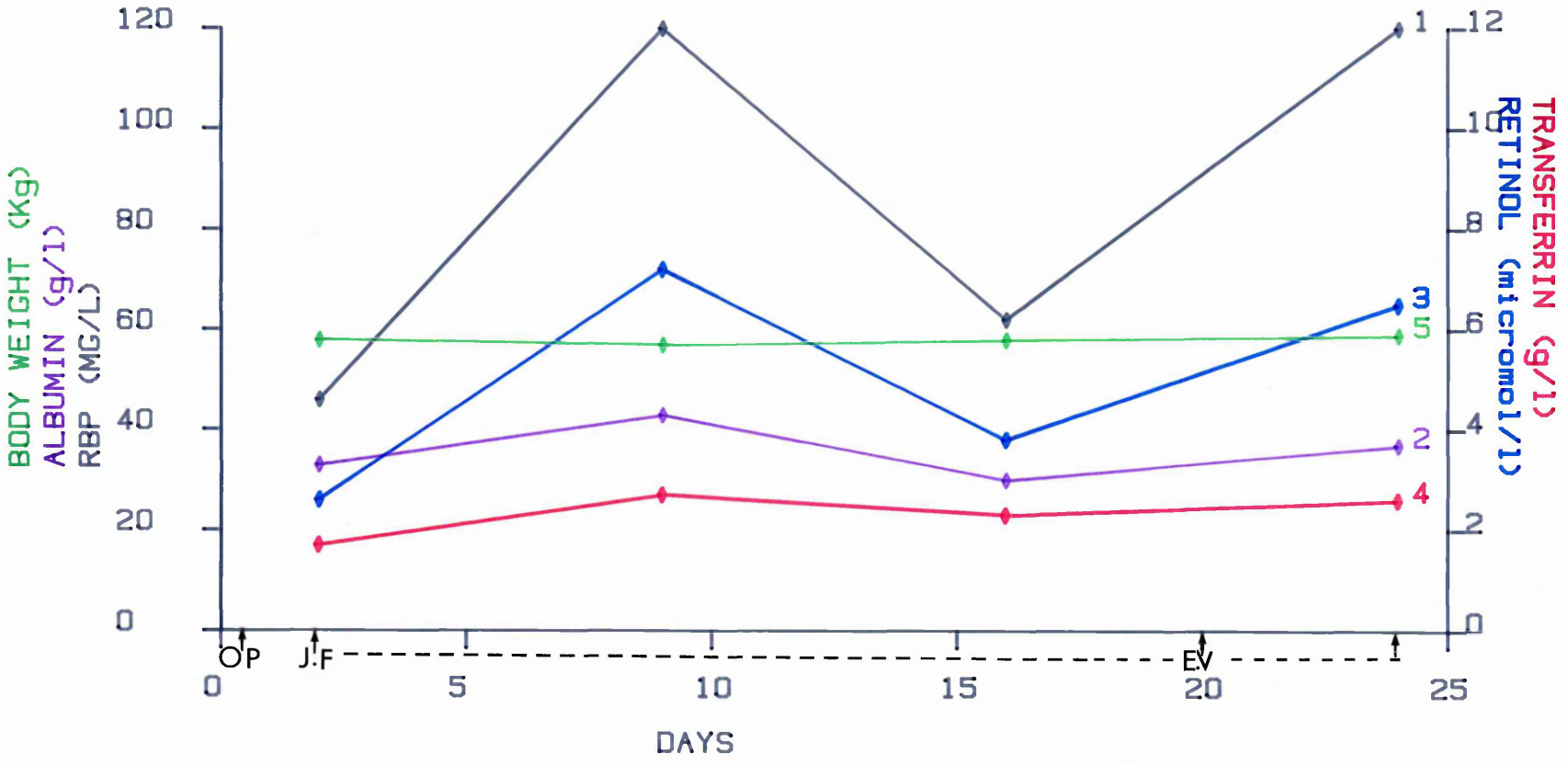
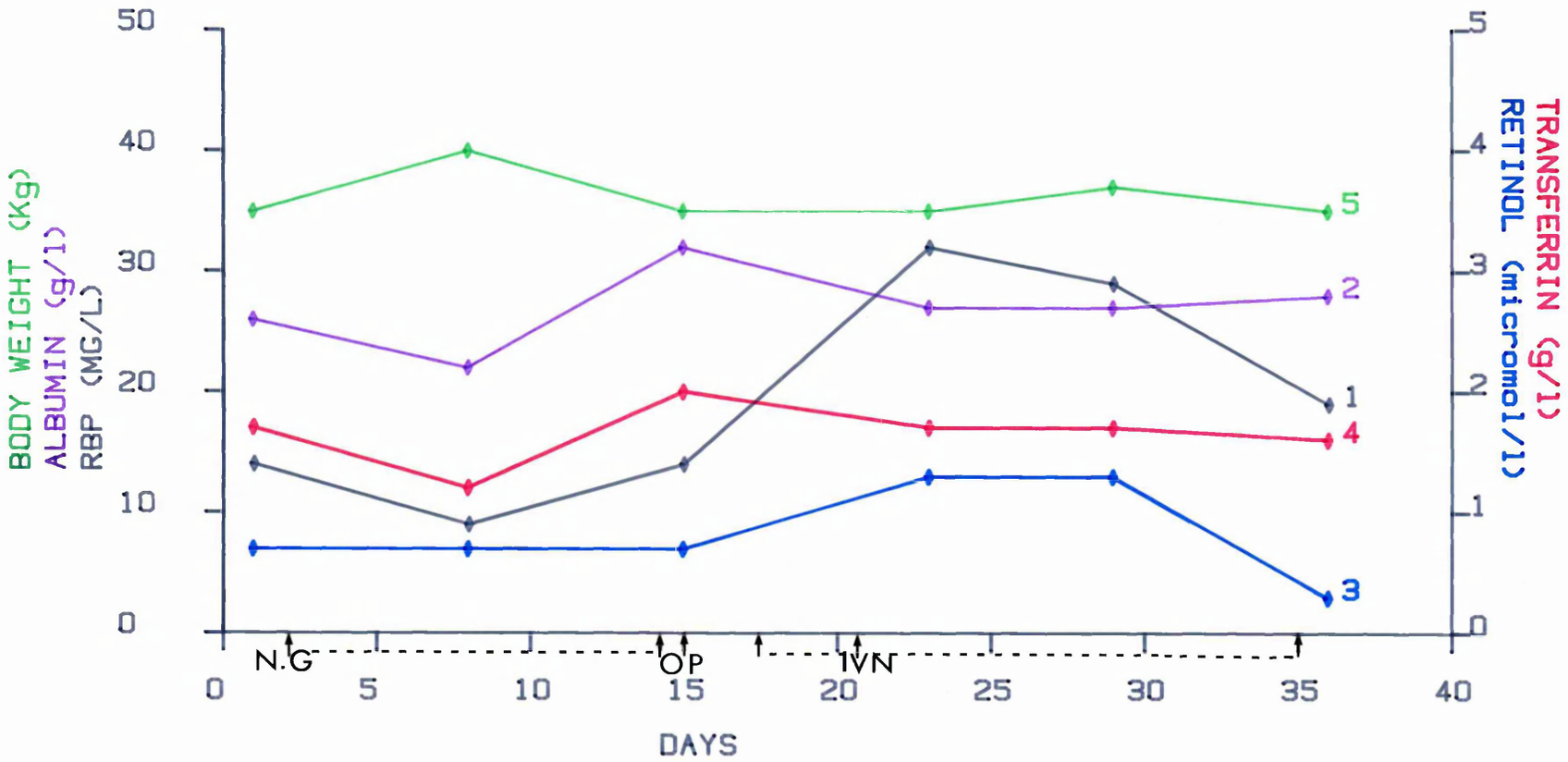

BODY WEIGHT (Kg)
ALBUMIN (g/l)
RBP (MG/L)
RETINOL (micromol/l)
TRANSFERRIN (g/l)
120
100
80
60
40
20
0
12
10
8
6
4
2
0
1
3
5
2
4
OP
J.F
EV
0
5
10
15
20
25
DAYS

Figure 42: Patient 10. The change in serum:

1) RBP (black)
2) Albumin (violet)
3) Retinol (blue)
4) Transferrin (red)
5) Body weight (green)

before and after surgical operation

NG = nasogastric feed
Op = operation
IVN = intravenous nutrition


BODY WEIGHT (Kg)
ALBUMIN (g/l)
RBP (MG/L)
TRANSFERRIN (g/l)
RETINOL (micromol/l)
50
40
30
20
10
0
5
4
3
2
1
0
5
4
3
2
1
N.G
OP
IVN
0
5
10
15
20
25
30
35
40
DAYS

et al 1975). Any function affecting RBP affects serum retinol. In patients there are different factors affecting the serum concentration of RBP.

Firstly, an acute-phase response which relates to different types of disease, operation or infection; where the fall in plasma retinol concentration in most patients may have been due to net protein catabolism (either reduced hepatic synthesis or increased catabolism) rather than temporary interruption of vitamin A supplements. This fact is supported by the fall in several plasma proteins measured including RBP. On the other hand, many proteins increase, eg CRP due to the acute-phase response. Dinarello, (1984) explains that tissue damage after injury leads to stimulation of macrophages to produce Interleukin-1 which causes liver to 'switch on' the synthesis of acute-phase proteins, with consequent increase in their concentration in plasma. Fleck et al (1985) suggested that the degree of tissue damage was evidently of importance in the activation of the acute-phase response, although the relationship is unclear. He explained why some concentrations of plasma proteins decrease after trauma, while others increase after showing an initial fall by a mechanism called a 'Two-compartment model'. This depends on many factors eg vascular permeability, molecular size and physical properties of the individual proteins. RBP has a small molecular weight (21,000), while albumin is 66,000 and transferrin is 80,000 and CRP is 105,000.

This may explain why RBP decreases before the other proteins after injury. Many factors may affect vascular permeability including Interleukin-1 and possibly other

endocrine effects. These factors may also affect synthetic rate. Therefore, an inverse relationship between CRP and RBP/retinol may be found in these patients, as was seen in the present study.

Aronsen et al (1972), Johansson et al (1972), Moody (1982) found in contrast to the acute-phase proteins there are many other proteins, whose concentrations fall after trauma and remain low for several days, eg albumin, transferrin, pre-albumin. In this study, these proteins also show such an effect. Fleck et al (1985) noted that the concentration of protein in plasma does not necessarily reflect its rate of synthesis or breakdown. Other factors such as vascular permeability can exert a greater influence on the concentration of proteins with a very rapid rate of turnover such as RBP, to which large and rapid changes in the rate of synthesis may lead within a day or two to significant changes in its concentration.

While the rise in serum retinol concentration with nutritional support in some patients was possibly due to a reduction in the acute-phase response, the other factors which affect RBP are vitamin A and protein nutritional status. The dependence of plasma vitamin A concentration on protein nutritional status makes it difficult to assess adequacy of provision of vitamin A. Lowry et al (1978) found that 2 patients continued to have low levels of plasma vitamin A for 10 days, despite receiving daily infusions of 720 ug and 860 ug vitamin A respectively. However, Nichoalds et al (1977) demonstrated that serum vitamin A concentrations can be raised to the normal range by giving 1,300 µg/day, whereas a similar regimen providing 1,000 µg/day failed to achieve normal serum vitamin A levels. Most of

our patients had positive N balance especially when RBP and retinol increased. Although our patients had vitamin A supplements the acute-phase response is greater and is usually the dominant effect. This appeared to be even stronger than in long term IVN patients, because the blood collected after injury and infection had higher CRP concentrations.

Peterson et al (1974), Smith et al (1975) suggested that malnutrition, disease and low diet protein, affect utilization, transportation and storage of vitamin A as well as the corresponding carrier protein from liver. Although these patients reported here were receiving nutritional support pre-and post-operation, the decrease in RBP in some cases may be due to trauma or sepsis which change the concentration of RBP rapidly, because of its short half-life and as a result of the acute phase response. Assessment of vitamin A status in such patients is therefore not possible using plasma measurements alone, and estimates of tissue content, eg from liver biopsy is probably necessary.

3.2.5) Studies on animals

3.2.5.1) Effect of vitamin A free diet on rats

Male rats (55-75 g) were studied in the Department of Surgery, Hope Hospital, Salford. They were initially fed on complete diet with a vitamin A supplement, until they were 3 weeks old to ensure relatively large initial liver vitamin A storage and to make them familiar with the experimental conditions. Forty rats were put on a vitamin A-free diet and 40 rats on complete diet (control). Five rats were killed from both deficiency diet group and complete diet group each week from week 1 to week 8. Rats were anaesthetised with fluothane and a blood sample collected by cardiac puncture into 0.3 ml heparin. Liver was removed and frozen until required for analysis. As part of another study 0.3 ml dextran was added per 1 ml blood, left at 37°C (in dark) for 30 min, then the plasma/dextran fraction was removed, centrifuged to remove leucocytes and then frozen in 0.5-1.0 ml aliquots until analysis. Table38 shows the composition of the diet fed to the animals and compared with those used by other workers.

Results

No difference was seen in weight gains of rats from control and deficiency groups as was shown in Figure 43. This finding confirms earlier observations by other investigators (Morita and Nakano, 1982). Changes in the serum levels of retinol in each dietary group are shown in Figure 44. Serum retinol levels decrease in both groups, but in the deficiency rats the decrease was greater and more rapid with a significant difference of $p < 0.001$ between the groups. The fall in the

TABLE 38

COMPOSITION OF THE DIET FED TO RATS, AND COMPARED WITH OTHER STUDIES

	1*	2	3**
Ingredient	g/kg	g	g/kg
Casein 'essentially vitamin free'	250	240	200+
Rice Starch (BDH)	660	659	700
Briggs salt mix (BP)	40	50	60
Corn oil	20	30	20++
Choline dry mix (20% choline chloride pre-mixed in corn starch)	-	10	-
Vitamin A (acetate)	3.0 mg	10	10
Water soluble vitamin	8.5 g	10	-
Crude-protein content (N x 5.7)	-	-	150

* Morita and Nakano (1982)
2 This study (1984)
** Muhilal and Glover (1974)
\+ Soya bean meal
++ Arachis oil containing (per kg) Vit D, E and phyloquinone

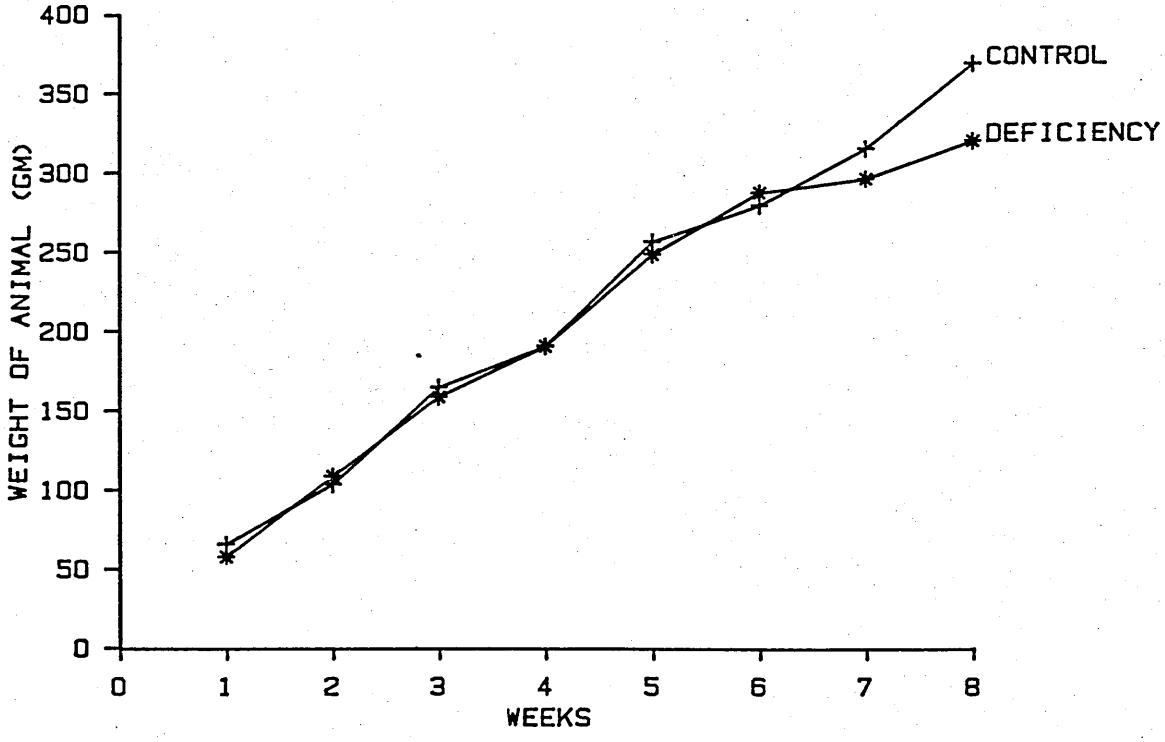


Figure 43: Weight of rats fed on a normal diet (+) and on vitamin A-free diet(*). The value represents the mean weight in each week.

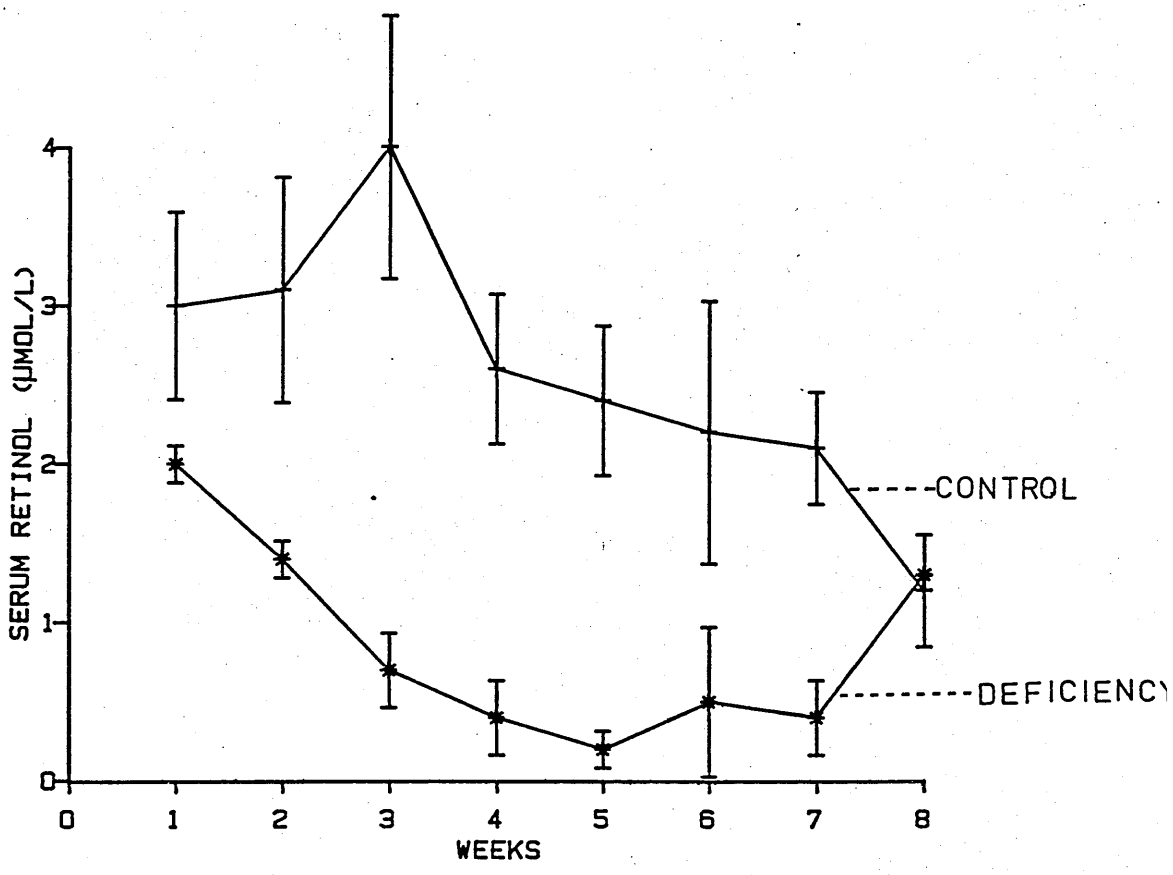


Figure 44: Serum retinol of rats fed on normal diet (+) and rats fed on vitamin A-free diet (*). The values are mean ± 1SD.

n = 5 for each group except week 8 deficiency group and control group n = 2

control group is not significant between week 1 and week 6. the increase after week 7 in the deficient group and the decrease in the control group may be artefacts, because at the end of week 8 only two rats were left in each group. In the control group, the total liver content of retinol gradually increased throughout the experiment, whereas it decreased rapidly in the deficiency groups and reached undetectable levels by week 2 (Figure 45).

Figure 46 shows the relationship between mean serum retinol and mean weight of animal in both control and deficiency groups. The heavier the animal becomes the lower the serum retinol levels.

Figure 47 shows the correlation between mean of serum retinol and total liver retinol. In general, the higher the amount of retinol stored in the liver, the lower the levels in serum.

Clinical signs of vitamin A deficiency were absent. The animals looked entirely healthy with no changes to their eyes or fur. The only complications were rather sudden death and the development of respiratory distress. Post-mortem examinations were done by the Veterinary School in Liverpool, England on a reasonable selection of the rats who died, and the cause of death was bronchopneumonia, although the organisms differed widely.

Discussion

The growth rate, liver and serum concentrations in this study agree with those of Morita and Nakano, (1982) who also found that animals grew well on a vitamin A-free diet.

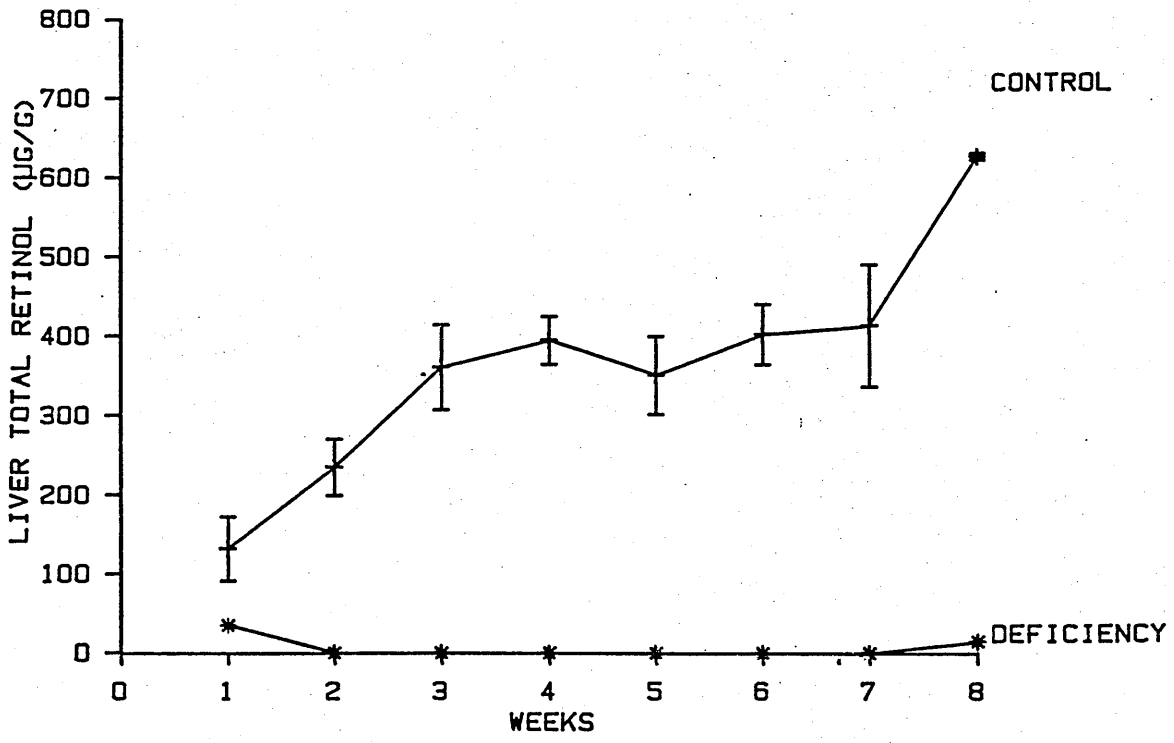


Figure 45: Liver total retinol in rats fed on normal diet (+) and rats on vitamin A-free diet (*). The values are mean ± 1SD

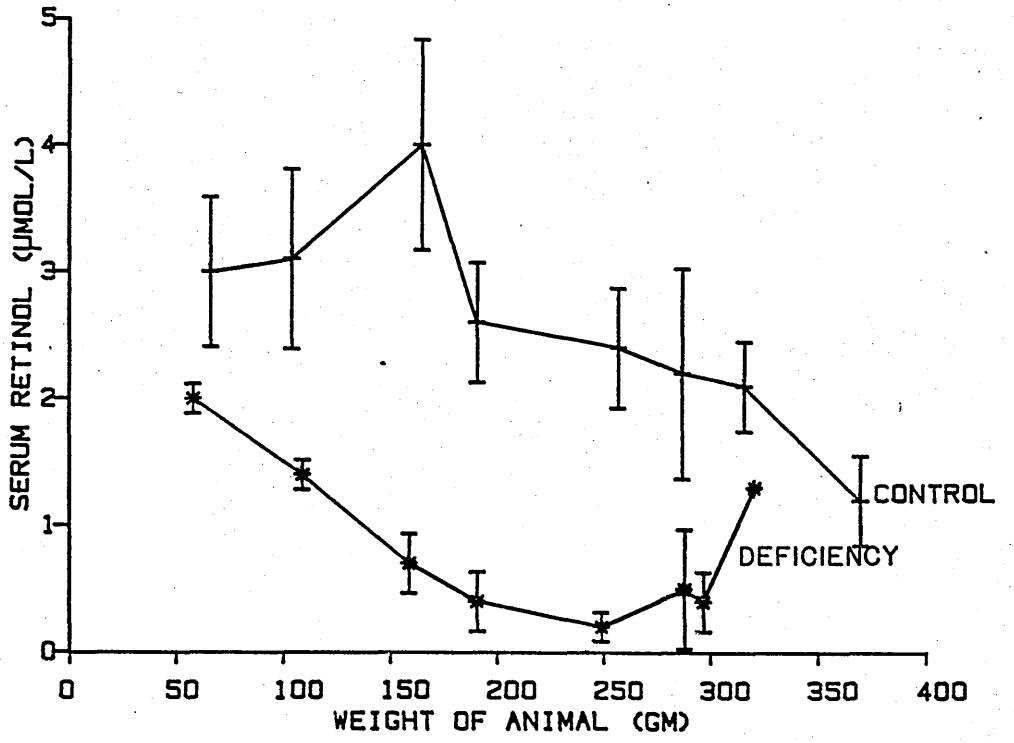


Figure 46: Relation between serum retinol and weight of animals on normal diet (+) and vitamin A-free diet (*). Values are mean ± 1SD.

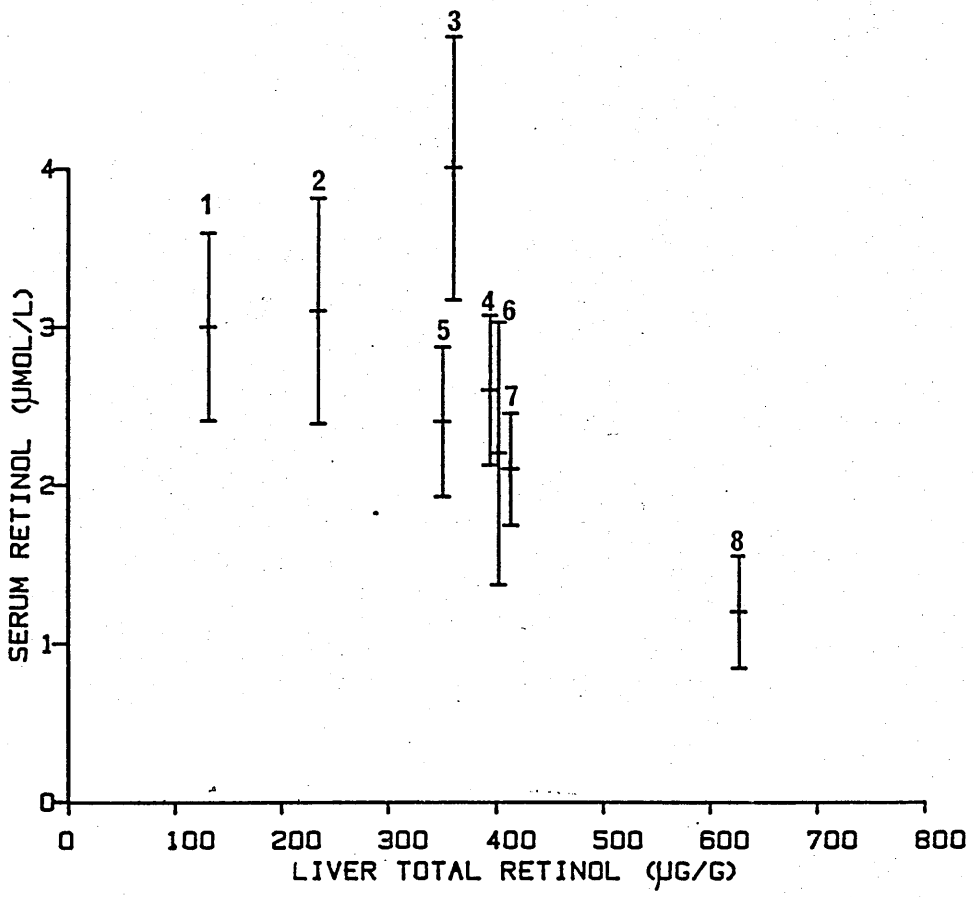


Figure 47: Serum retinol (mean±1SD) related to mean total liver retinol in control rats. Numbers 1-8 correspond to the number of weeks on control diets.

Lewis et al (1972) believed that plasma tends to resist to a certain extent, any changes in its vitamin A concentration despite wide variations in the amount stored in the liver. While stores remain in the liver, retinol is maintained in plasma and resists any change in concentration in the diet. Although there was a slight tendency for plasma retinol to fall in the control animals, overall the concentration was well maintained during the study. The fall in plasma retinol appeared to be related to an accumulation of retinol in the liver, whilst the animal was growing. In the deficiency groups the plasma level decreased to a low level when the amount in the liver was undetectable.

Glover et al (1947) found that plasma vitamin A levels in rats are proportional to the concentration of vitamin A alcohol in the liver, and not proportional to the total liver stores of vitamin A which consist mainly of esters. Plasma vitamin A levels are maintained near normal, even when liver stores approach exhaustion.

Ganguly and Krinsky (1953) used several groups of male rats receiving various diets and supplements. Despite the variation of the liver vitamin A alcohol, the plasma vitamin A alcohol remained constant. Only in the group of rats 2 months old fed a vitamin A-free diet were the liver vitamin A alcohol and plasma vitamin A undetectable. The results in this study are similar to those of Robison et al (1980) who found that after 14 weeks of deficiency of vitamins A and E, plasma and liver vitamin A were undetectable. In contrast, in the present study, liver vitamin A was undetectable after only 2 weeks on vitamin A-free

diet in 3 week old rats. The explanation for this is not clear, but it may be related to the rapid rate of growth of these animals.

Another explanation of this is that the liver plays a central role in maintaining blood retinol levels. Vitamin A is stored in liver for supplying tissue needs, when the dietary intake of vitamin A decreases. The liver also synthesises retinol carrier proteins to circulate stored retinol to the tissues. Morita and Nakano (1982) examined the concentration of vitamin A in kidney and other tissues at the same times they measured vitamin A in liver and plasma. They found that vitamin A concentration increased in the kidney. They suggested that perhaps the kidney was playing an important role in storing the vitamin A, when the exogenous supply to animals decreased. High (1954); Peterson et al (1973), suggested that vitamin A accumulated in this organ may be re-circulated into blood. They suggested also, that retinol may be released from its carrier protein in the kidney, where the carrier protein is catabolised, and then re-circulated to the blood. Kidney was not examined in our study.

Resistance of these animals to infection was reduced and this may be related to the bronchopneumonia and death (Section 1.7).

Scrimshaw et al (1968), Gruber et al (1976), suggested that the susceptibility to infection in vitamin A-deficiency appears to be due at least in part to an inability of epithelial cells to synthesise the secretory component of secretory-IgA, such that local immune responses are impaired.

It is interesting to note that despite a vitamin A-free diet, rats could still grow at a normal rate for the duration of the study. However, in such animals, liver stores are rapidly depleted but no physical signs were apparent up to 8 weeks. Since these rats had a low serum and liver vitamin A, it is probable that a deficiency state would develop if the study was continued.

3.3) CANCER STUDIES

3.3.1) Studies on patients

3.3.1.1) Concentration of serum retinol, cholesterol and triglyceride and relation to colorectal cancer

A random sample of 70 persons with no known ill-health and between the ages of 40 and 86, born in the Eastern District of Glasgow were studied. A further 70 patients with a history of colorectal cancer, and born in the same district were also studied. A fasting blood sample was taken from both groups. In the colorectal group the blood samples were taken between 7 days and 12 years (median one year) post operations. At the same time a medical history was obtained, including age, sex, height, weight, any personal or family history of cancer, heart disease, diabetes or hypertension, occupation, smoking and drinking habits, exposure to drugs and medication (carried out by local General Practitioner). Blood samples were analysed for serum retinol, total cholesterol and triglyceride.

Results

The 70 patients had mean serum retinol levels slightly lower than control with $p < 0.01$ (t-test). The difference between males with treated cancer and controls was also significant ($p < 0.01$). There was no significant difference between the serum retinol concentration of females with treated cancer, and controls (Table 39). Male controls had a higher serum retinol value than females ($p < 0.05$). There was no significant difference in serum retinol between smoking (20 cigarettes/day) and non-smoking in control subjects (Table 40).

TABLE 39

SERUM RETINOL IN SUBJECTS WHO HAD BEEN TREATED FOR CANCER AND CONTROL (mean ± 1SD)

Subjects	No. of Cancer patients	No. of control patients	Cancer (μmol/l)	Control (μmol/l)
Total	70	70	2.7±1.1	3.2±0.9
Male	35	27	2.7±1.2	3.4±1.1
Female	35	31	2.6±1.0	2.9±0.8

TABLE 40

COMPARISON BETWEEN SERUM RETINOL OF SMOKING AND NON-SMOKING CONTROLS (mean ± 1SD)

Subject	No. of smoking subjects	No. of non-smoking subjects	Smoking (μmol/1)	Non-smoking (μmol/1)
Total	26	16	3.0±0.9	3.4±1.0
Male	14	6	3.3±0.9	3.7±1.3
Female	12	10	2.8±0.7	3.2±0.8

TABLE 41

THE EFFECT OF AGE ON SERUM RETINOL IN CANCER PATIENTS AND CONTROLS (mean ± 1SD)

Age	No. of Cancer patients	No. of Control patients	Cancer (μmol/1)	Control (μmol/1)
< 50	4	24	2.7±1.2	3.1±1.0
51-60	8	22	2.6±1.1	3.3±1.0
> 60	31	12	2.4±1.1	3.0±0.7

Age also did not affect the results since there was no significant difference between different age groups (Table 41).

Tables 42-44 show the comparison between serum cholesterol in cancer cases and control patients and the effect of smoking and age. No significant difference was found between cancer cases and controls in any comparison.

Tables 45-47 show no significant difference in serum triglyceride between cancer patients and control and no effect of sex, smoking and age respectively.

Figures 48-53 show the correlation between serum retinol and total cholesterol, serum retinol and triglyceride and triglyceride and total cholesterol in both control and cancer patients respectively. Poor correlation was found in both groups (Table 48).

Correlation of $r = 0.5$ ($p < 0.001$) was found between total serum cholesterol and serum retinol in cancer patients.

Patients who had metastases in liver had significantly lower serum retinol and cholesterol than cancer patients without metastases (Figures 54,55 and Table 49).

Figure 56 shows the lack of relationship between serum retinol and the time of diagnosis ($r = 0.2$) in all cancer patients, There is a poor correlation between serum retinol, total cholesterol and the time from diagnosis $r = 0.6$, $r = 0.5$ respectively in metastases cases. Most patients with metastases show low serum retinol and cholesterol, with significant difference of $p < 0.01$ from no metastases and control. However, their blood samples had mainly been taken 7-15 days post-operation. On the other hand, most of

TABLE 42

COMPARISON IN TOTAL SERUM CHOLESTEROL BETWEEN CANCER PATIENTS AND CONTROL (mean ± 1SD)

Subject	No. of cancer patients	No. of control subjects	Cancer (mmol/l)	Control (mmol/l)
Total	64	63	6.0±2.1	5.9±1.1
Male	32	27	5.7±2.3	5.8±0.98
Female	32	31	6.2±1.8	6.0±1.2

TABLE 43

COMPARISON BETWEEN TOTAL SERUM CHOLESTEROL IN SMOKING AND NON-SMOKING CONTROLS (mean ± 1SD)

Subject	No. of smoking	No. of non-smoking	smoking (mmol/l)	non-smoking (mmol/l)
Total	26	16	5.8±1.1	6.2±1.1

TABLE 44

EFFECT OF AGE ON TOTAL SERUM CHOLESTEROL BETWWEN CANCER PATIENTS AND CONTROL (mean ± 1SD)

Age	No. of cancer patients	No. of control	Cancer (mmol/l)	Control (mmol/l)
< 50	2	24	4.9±1.2	5.8±1.1
51-60	7	22	6.0±2.7	6.1±1.3
> 60	29	12	5.9±2.5	5.7±0.8

TABLE 45

COMPARISON OF SERUM TRIGLYCERIDE BETWEEN CANCER PATIENTS AND CONTROL (mean ± 1SD)

Subject	No. of Cancer patients	No. of Control	Cancer (mmol/l)	Control (mmol/l)
Total	64	63	1.9±0.9	1.6±1.2
Male	32	27	1.9±1.0	1.5±0.6
Female	32	31	1.9±0.8	1.6±1.5

TABLE 46

EFFECT OF SMOKING ON SERUM TRIGLYCERIDE ON CONTROL (mean ± 1SD)

Subject	No. of smoking	No. of non-smoking	Smoking (mmol/l)	Non-smoking (mmol/l)
Total	26	16	1.4±0.6	1.4±0.6

TABLE 47

COMPARISON OF THE EFFECT OF AGE ON SERUM TRIGLYCERIDE BETWEEN CANCER AND CONTROL (mean ± 1SD)

Age	No. of Cancer patients	No. of Control	Cancer (mmol/l)	Control (mmol/l)
< 50	2	24	1.6±0.0	1.3±0.6
51-60	7	22	1.8±0.8	1.8±1.7
> 60	29	12	1.7±0.9	1.6±0.7

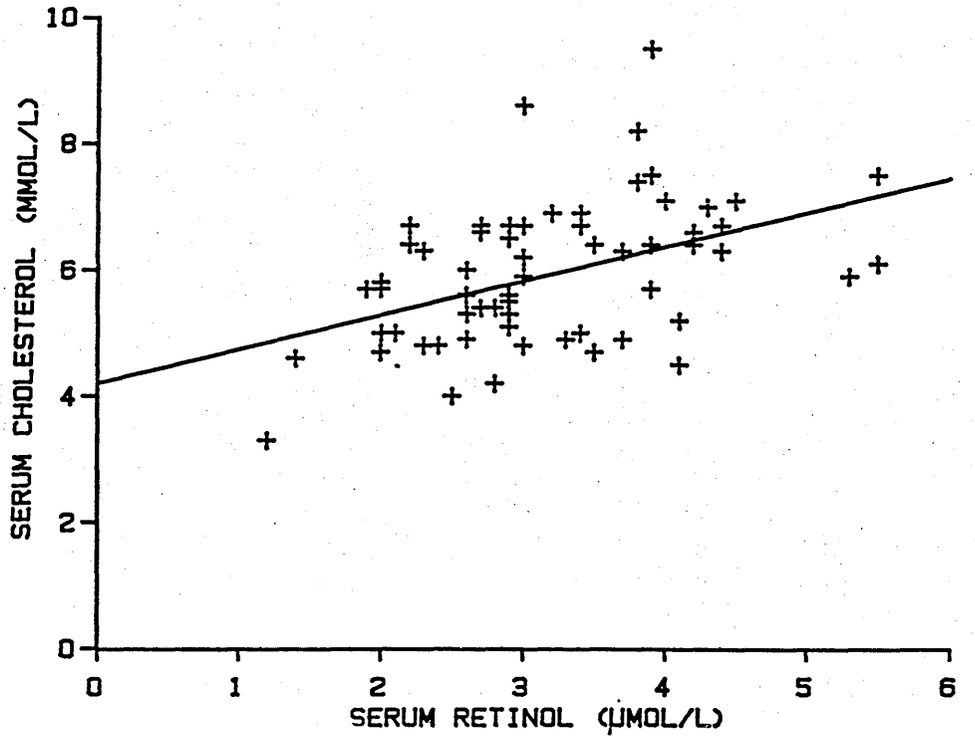


Figure 48: Correlation between serum retinol and cholesterol in control patients.

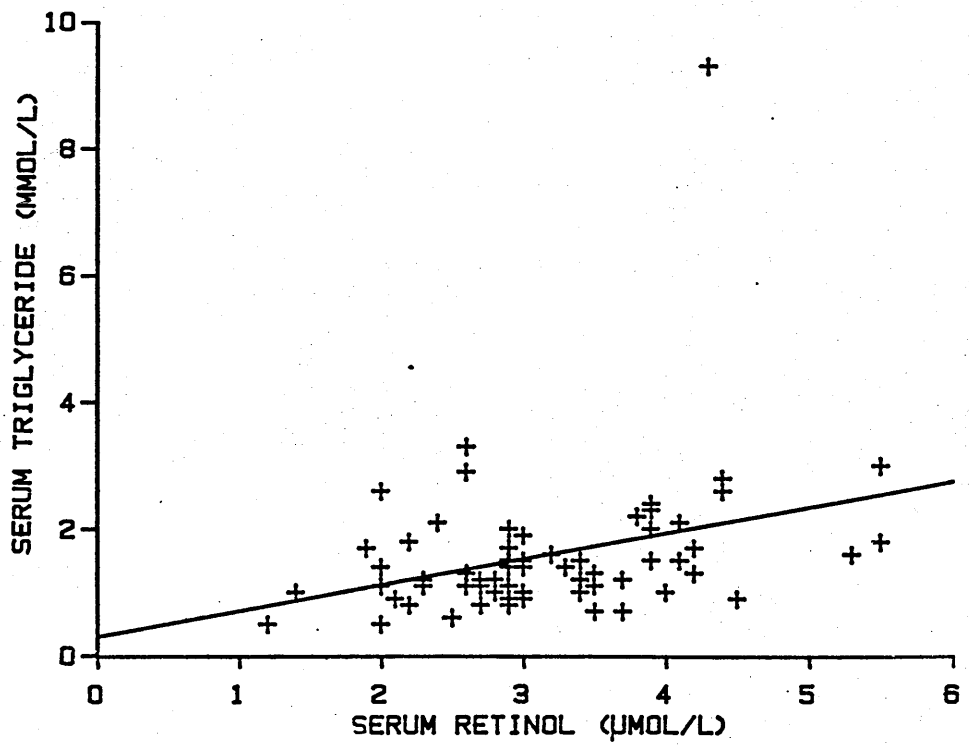


Figure 49: Correlation between serum retinol and triglyceride in control patients.

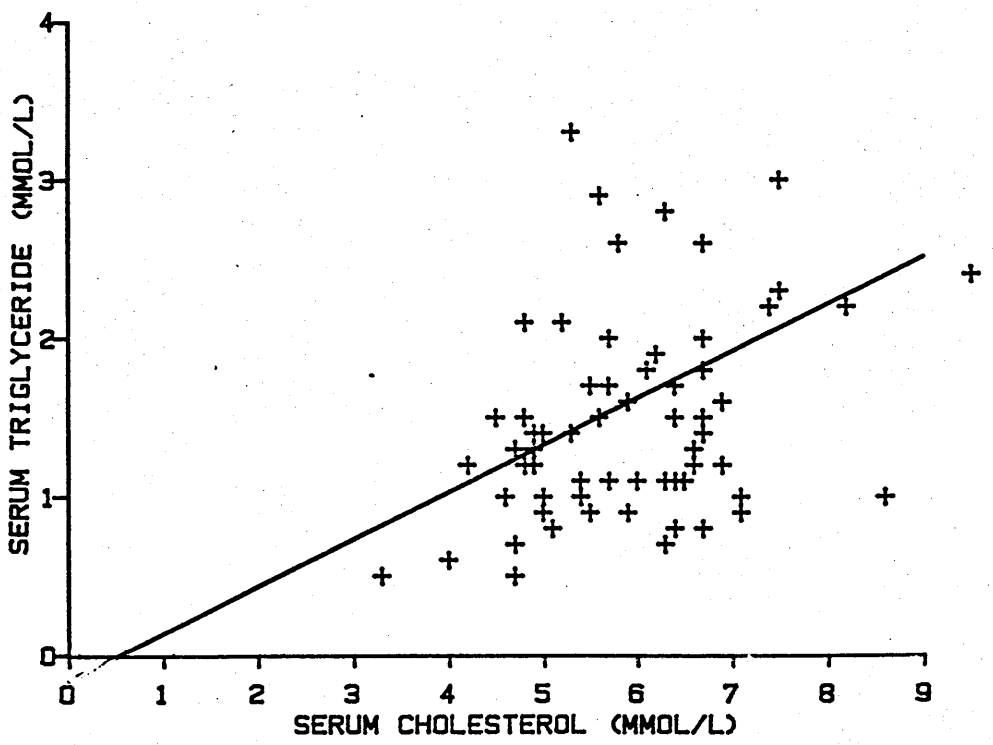


Figure 50: Correlation between serum cholesterol and triglyceride in control patients

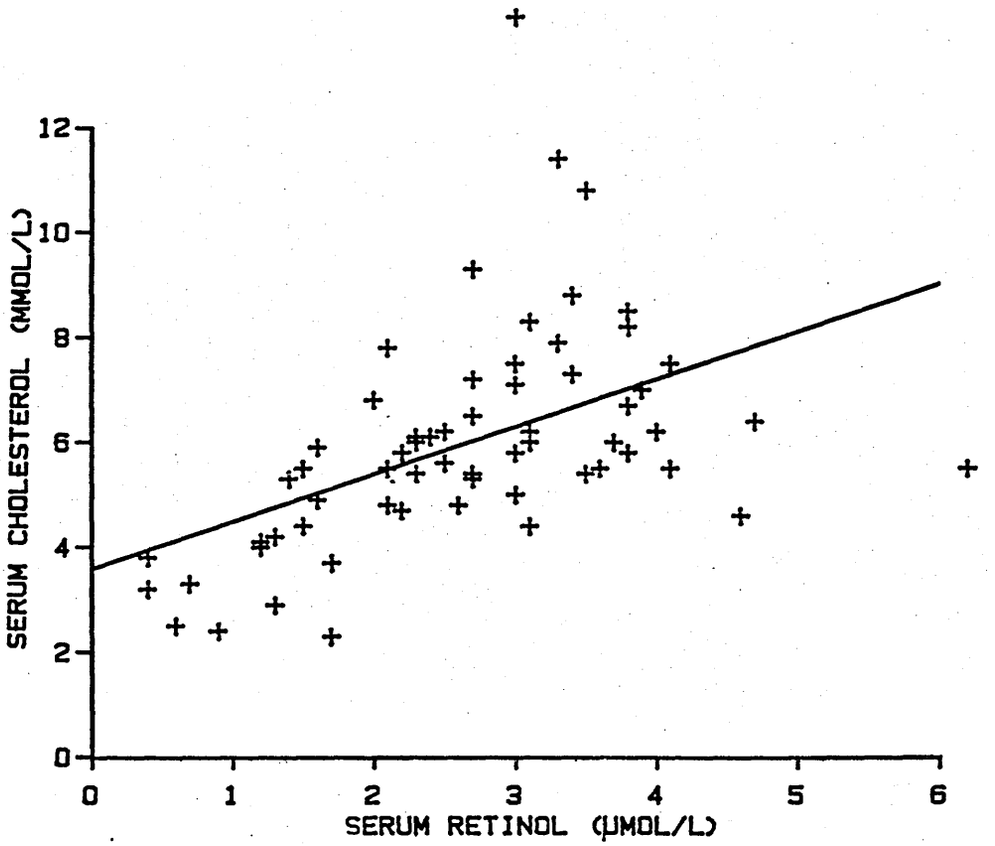


Figure 51: Correlation between serum retinol and cholesterol in colorectal cancer patients.

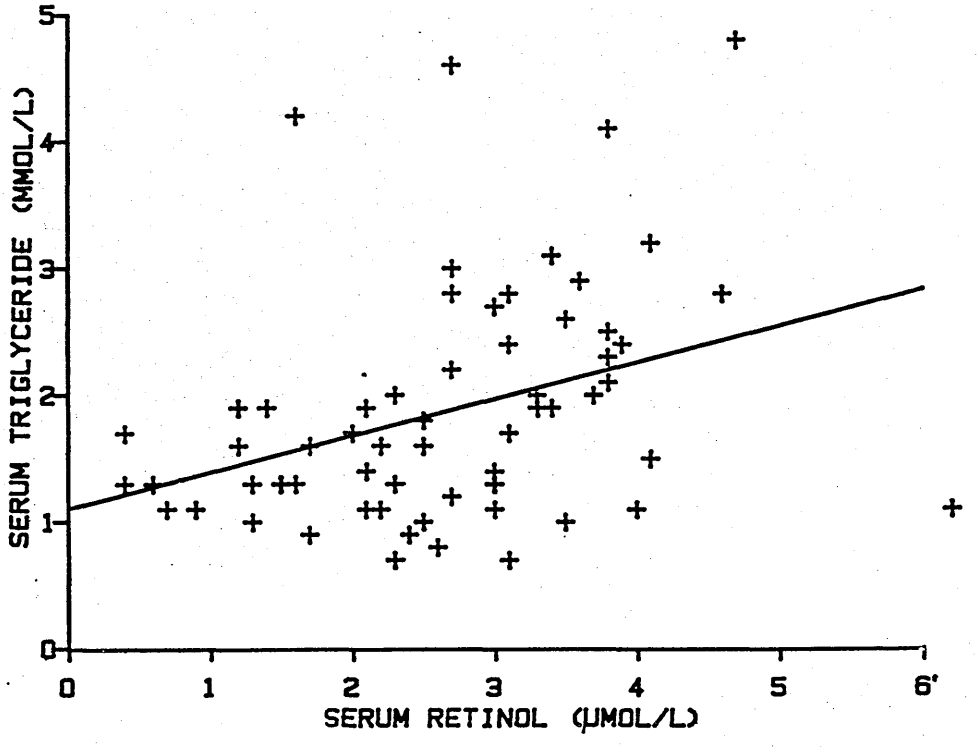


Figure 52: Correlation between serum retinol and triglyceride in colorectal cancer patients.

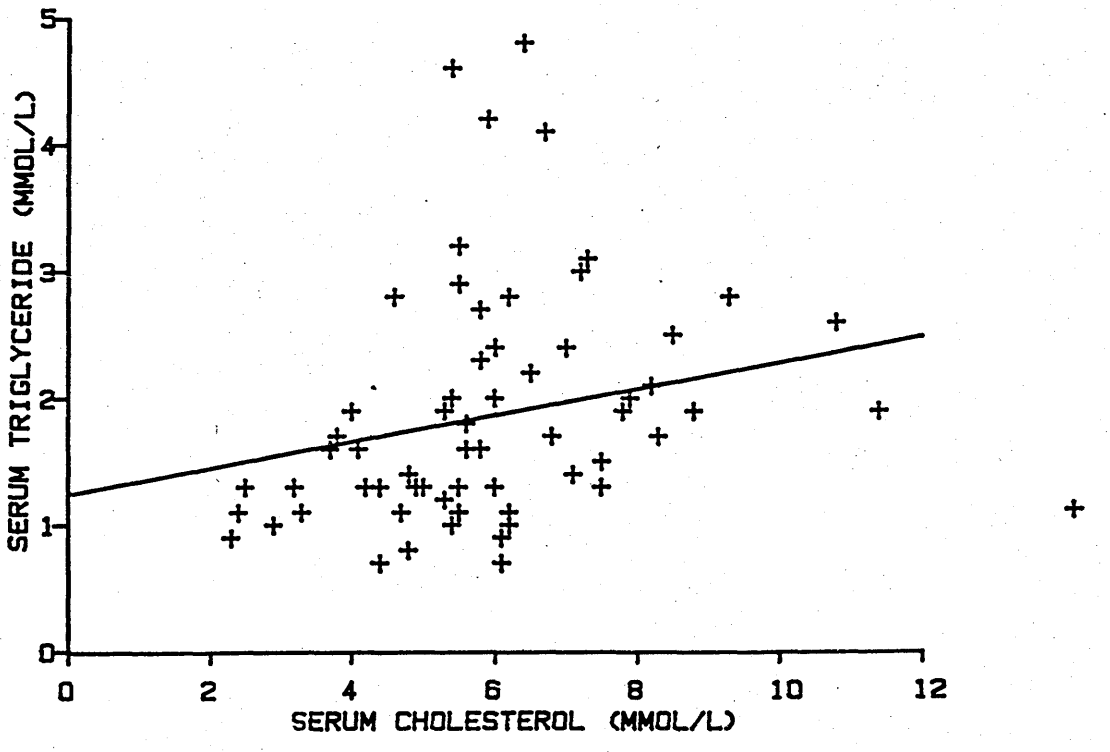


Figure 53: Correlation between serum cholesterol and triglyceride in colorectal cancer patients

TABLE 48

CORRELATION BETWEEN SERUM CHOLESTEROL, TRIGLYCERIDE AND SERUM RETINOL IN BOTH CANCER PATIENTS AND CONTROL PATIENTS

Cases	Cholesterol v Retinol	Triglyceride v Retinol	Cholesterol v Triglyceride
Control	$r = 0.4$ $p < 0.001$	$r = 0.3$ $p < 0.01$	$r = 0.3$ $p < 0.02$
Cancer	$r = 0.5$ $p < 0.001$	$r = 0.2$ $p < 0.05$	$r = 0.2$ $p < 0.05$

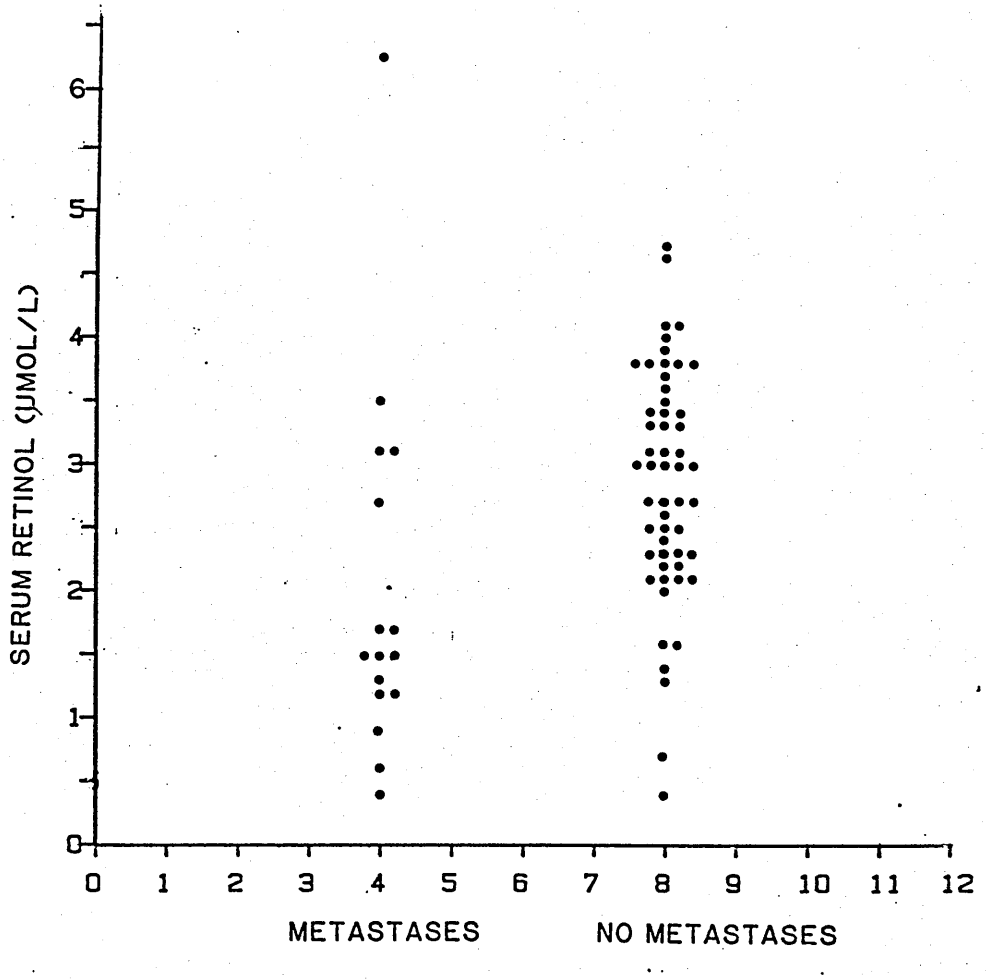


Figure 54: Difference in serum retinol in colorectal cancer patients with or without metastases

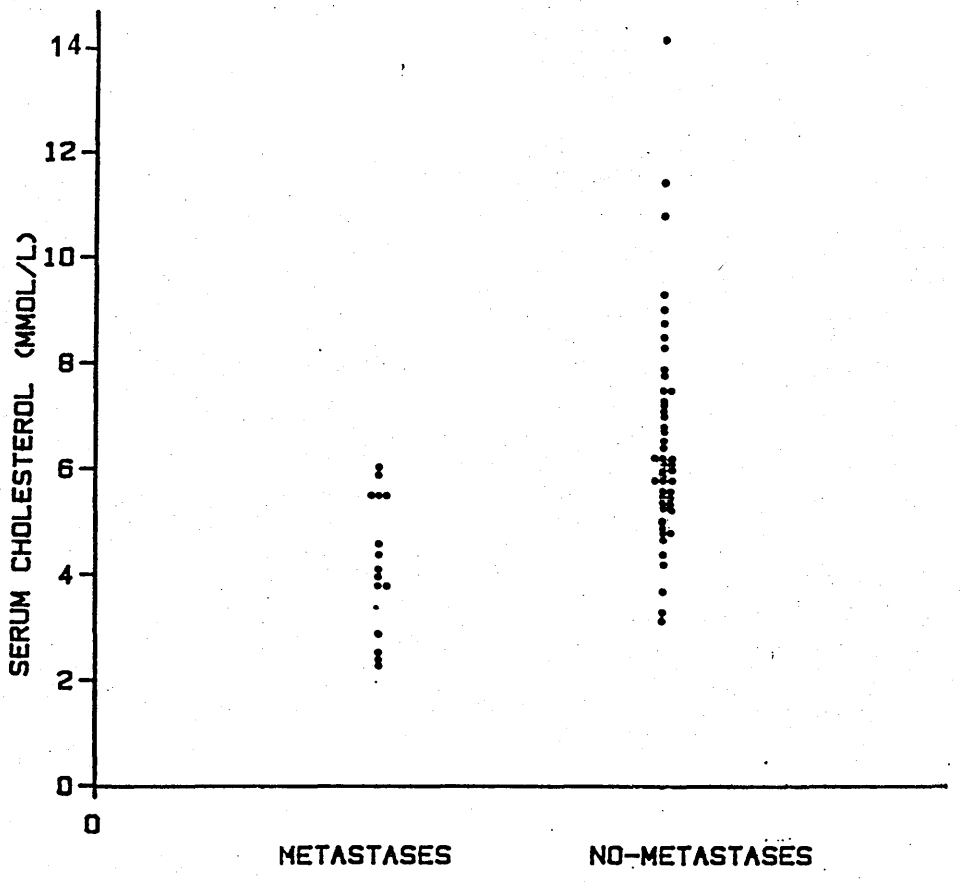


Figure 55: Difference in serum cholesterol in colorectal cancer patients with or without metastases

TABLE 49

COMPARISON OF SERUM RETINOL AND CHOLESTEROL BETWEEN METASTASES AND NON-METASTASES (Mean ± 1SD)

Subject	Metastases	Non-Metastases	
Retinol (μmol/l)	1.6 ± 1.6	2.5 ± 0.9	$p < 0.01$
Cholesterol (mmol/l)	4.1 ± 1.3	6.5 ± 2.0	$p < 0.001$

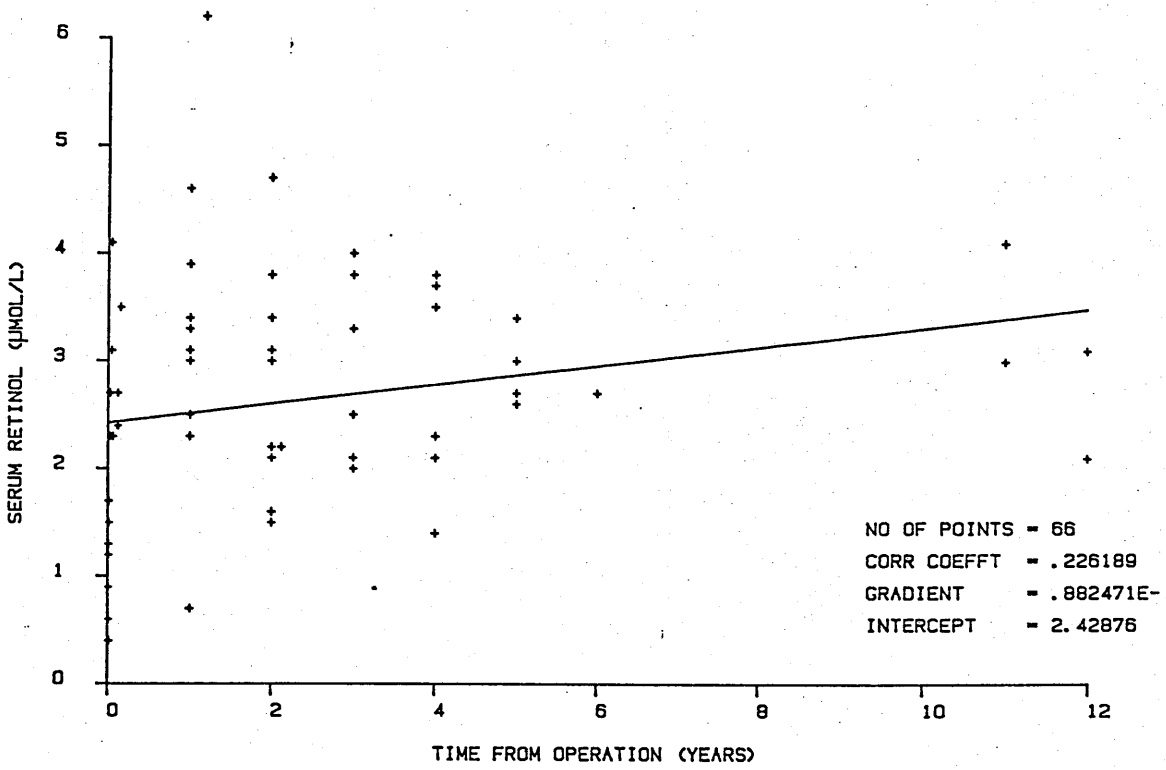


Figure 56: Regression of serum retinol on time from operation in patients with colorectalcarcinoma

the patients without metastases at operation had samples taken later in the post operation period (Figure 59) and there was no correlation between serum retinol and time after operation, and with no significant difference of control.

Discussion

Taken overall there appears to be a relationship between low serum retinol levels and patients having had a previous operation for colorectal cancer. Table 39 shows that cancer cases had lower serum retinol than control ($p < 0.01$) and this relationship is independent of age, smoking habit or serum triglyceride concentration. Liver metastases were found to be associated with lower serum retinol levels and lower cholesterol if compared to 'no metastases' patients. However, closer examination of the patient population shows that the metastases group of patients were primarily sampled in the early post-operative period, whereas the non-metastases groups were sampled over a more prolonged period (up to 12 years) following operation. This reflects the much better prognosis in the 'no metastases group'. The main reason for the low serum retinol in the metastasis group is therefore probably an acute phase response to the surgery. When the samples taken within 1 month of operation are excluded, then the serum retinol on the remaining cancer patients is not significantly different from the control population. Patients who have previously developed a colorectal cancer do not therefore have a long-term underlying abnormality in serum retinol. It is important to know that 'no metastases' group of patients had already undergone removal of their carcinoma, and were believed to be free of neoplastic

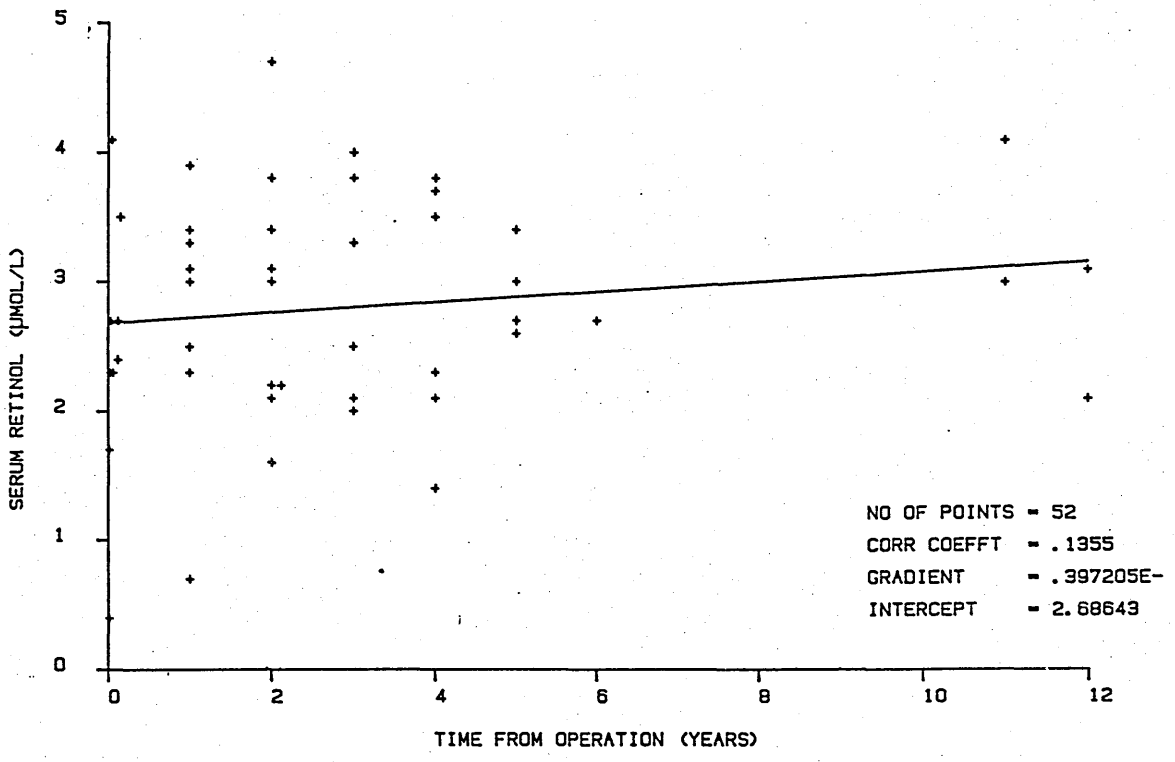


Figure 59: Regression of serum retinol on time from operation in patients with colorectal carcinoma without metastases

disease following surgery, when the blood samples were collected. In addition, these patients were not undergoing any other kind of therapy, eg chemotherapy or radiotherapy. There was a correlation of r = 0.6 and r = 0.5 between low serum retinol and low cholesterol respectively with time of diagnosis. These findings appeared in blood samples collected 7 to 15 days post-operatively. Basu et al (1985) investigated serum retinol levels and RBP in post-operative colorectal cancer patients. He found that plasma retinol levels in the post operative disease-free colorectal cancer patients were lower than those in normals. This finding was confirmed in the present study (Table 39). Furthermore, the initial plasma retinol level in conjunction with RBP was found to be lower in patients who subsequently had cancer recurrence than in those who remained free of apparent cancer. They found low serum retinol levels and low RBP and a correlation between poor metabolic vitamin A status and subsequent relapse. All these observations may have prognostic and predictive significance in colorectal cancer.

The difference in serum retinol between cancer patients and controls in the early post operative period appeared to be greater in males than in females. A similar observations was found by Wald et al (1980) in patients with lung cancer.

Ten out of eleven retrospective studies (Kummet and Meyskens, 1983) concluded that low levels of serum retinol existed in the cancer population. The difference between cancer cases and controls ranged from 0.1 µmol/l to 1.7 µmol/l with an average of 0.7 µmol/l.

The only correlation we found was between serum retinol and total cholesterol (r = 0.5) in cancer patients (Table 48) and this correlation disappeared if samples taken within one month of operation were excluded. These cancer patients already had surgery, but some had low serum cholesterol especially those who had metastases and had blood collected 7-15 days post operations. This could be a response to the cancer process. In previous studies, the relationship with cancer was stronger with serum retinol than with cholesterol. Kark et al (1982) suggested that the association with cholesterol might be secondary.

It is however more likely that the acute phase response accounts for the lower serum cholesterol observed in patients with metastases. Serum apolipoprotein B is known to decrease as a result of operation (Ballantyne et al 1979). Therefore it cannot be concluded from this study whether a low serum cholesterol is associated with metastases.

Triglyceride did not have any association with either serum retinol or cholesterol or occurrence of cancer.

Further studies were carried out (in the Department of Genetics, University of Aberdeen) on the genotype of lipoprotein in controls and relating this to the risk of cancer. There was no relationship with age, sex (except HDL2 in females). For the same genotype there were small, but statistically significant relationships with cigarettes and/or alcohol consumption. The most remarkable feature found was a negative correlation between serum retinol and HDL2 concentration in females. Why a relation-

ship was found between HDL2 and retinol in females, although there was no such relationship found in males, may be due to other factors in females affecting such a relationship.

Adams et al (1985) studied the association of lipoprotein cholesterol with vitamin A. They investigated the relationship between serum carotene and cholesterol in multiracial groups of college volunteers. These results were consistent with the hypothesis that the association between serum cholesterol concentration and cancer may be the result of a relationship between lipoprotein cholesterol concentration and vitamin A metabolism. As indicated, the association is likely to be indirect between low serum cholesterol and cancer risk. One possible mediator is carotene. The lipoprotein subfractions are involved in the transport of carotene. Simpson and Chichester (1981) found more than 50% of the carotenoids in the low-density lipoproteins (LDL) and the remainder in HDL. The evidence of a relationship between cholesterol concentrations and cancer has implicated primarily total cholesterol and LDL cholesterol and not HDL cholesterol.

Undoubtedly, multiple factors contribute to the development of cancer. Additional epidemiological and experimental studies are needed to assess the relationships among vitamin A, cholesterol and cancer risk to clarify the mechanisms involved, and to distinguish the role of vitamin A in the aetiology of cancer. Further study is required to elucidate the exact relationship between vitamin A and its transport protein with epithelial and non epithelial tumours (Tyler et al 1985).

From the present study, it cannot be deduced whether impaired vitamin A status predisposes to development of cancer of colon or to metastases occurring, or whether the cancer/metastases causes the change in plasma retinol concentration. Although the cancer group appeared to have no long-term abnormality in retinol status, the association of retinol with HDL2 in females is worthy of further study.

3.3.1.2) Determination of serum retinol in patients with oral diseases

Patients from the Dental Hospital out-patient department (Glasgow) with disease of the mouth were studied. Biopsy of the mouth lesion was taken at the same time as a venous blood sample. Histology indicated that 19 patients had oral cancer, 11 leukoplakia of the oral cavity and 7 Crohn's disease. Forty-five individuals with no oral lesion were studied to establish the normal levels of serum retinol and vitamin E in this population (Table 50).

Figures 60 and 61 and Table 50 shows mean serum retinol and vitamin E levels in each group. There was no significant difference in serum retinol between patients with oral cancer or leukoplakia and normal subjects, while there was a significant difference between patients with Crohn's disease and normal subjects ($p < 0.001$). For serum vitamin E there was a significant difference between normal and cancer patients ($p < 0.01$; Mann-Whitney test).

If we group these cases according to whether the serum retinol is below or above 2 SD of normal subjects, 20% of cancer cases show low values, whereas 57% of Crohn's disease patients had serum retinol levels below the normal range (Table 51).

Discussion

The results obtained show that there were no significant differences in serum retinol between oral cancer or leukoplakia and normal cases, although it was expected that lower levels of serum retinol may have been associated with oral cancer and leukoplakia (a lesion which might progress to cancer).

TABLE 50

SERUM RETINOL AND VITAMIN E IN DIFFERENT DISEASES OF ORAL CAVITY (mean ± 1SD)

Case	No. of cases studied	Serum retinol (μmol/l)	Vitamin E (μmol/l)
Normal	45	2.58±0.69	29.0±49.2
Cancer	19	2.39±1.24	11.7±9.3
Leukoplakia	11	2.45±0.93	22.3±8.9
Crohn's Disease	7	1.5±0.55	18.7±5.5

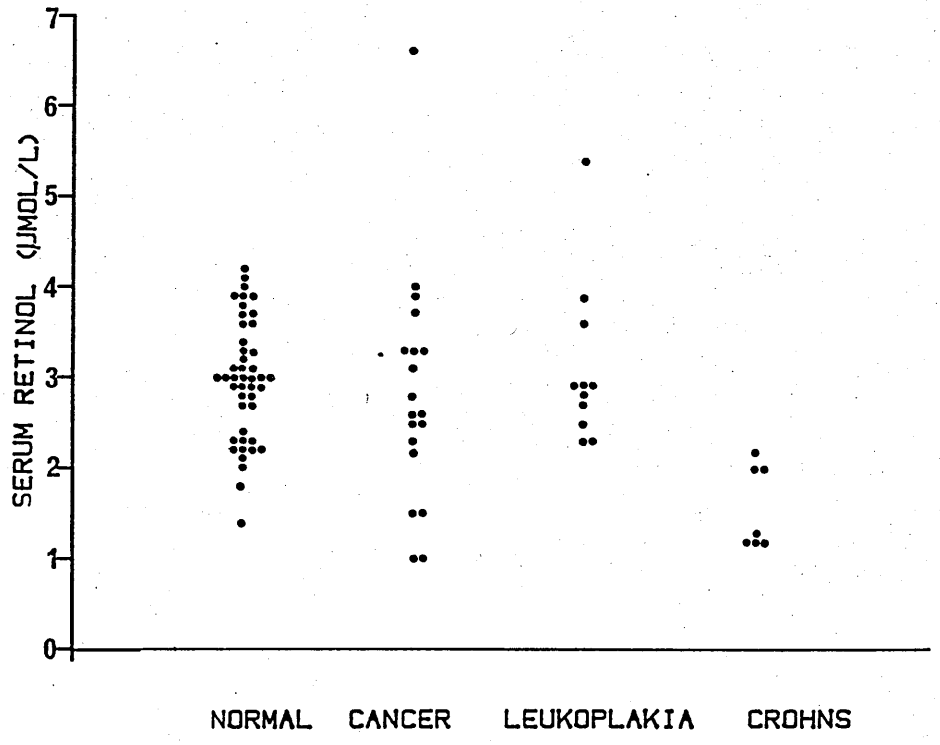


Figure 60: Serum retinol in patients with disease of oral cavity

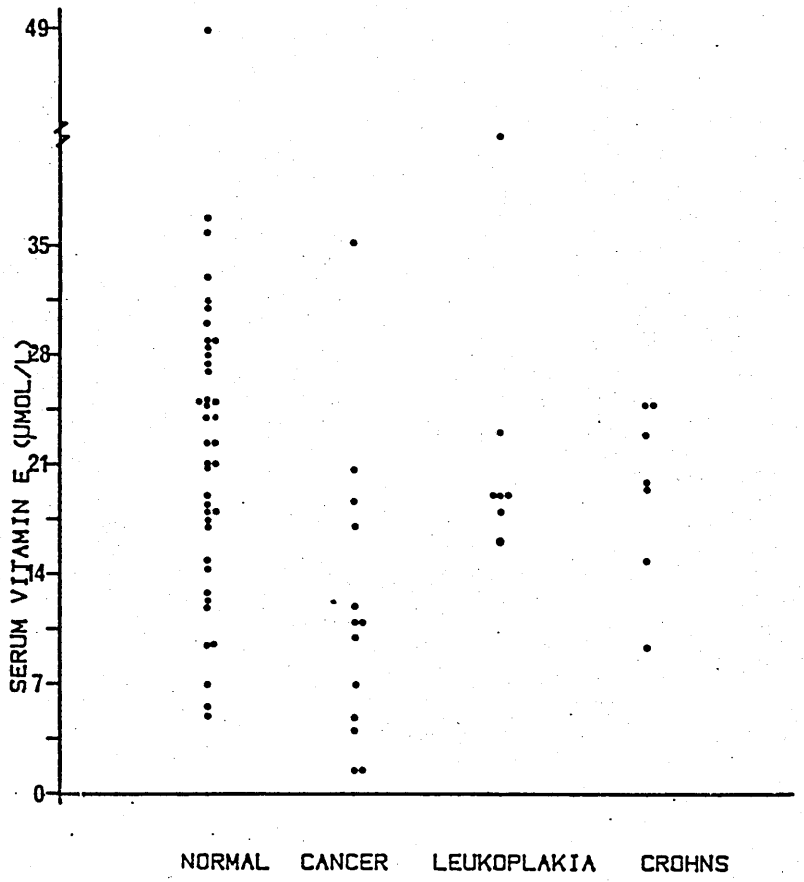


Figure 61: Serum vitamin E in patients with disease of oral cavity

TABLE 51

INCIDENCE OF ABNORMAL SERUM RETINOL

Case	Low	Normal	High
Normal	2(4%)	43(96%)	-
Cancer	4(21%)	14(74%)	1
Leukoplakia	0	10(90%)	1
Crohn's Disease	4(57%)	3(43%)	-

Moore (1967); Toyoshima and Leighton (1975) described that the deficiency of vitamin A causes keratinization or squamous metaplasia of mucous epithelia in which mucous membrane change from single layer of mucin-secreting and ciliated epithelium to multiple layers of epithelial cells, with overlying keratin, resembling those of skin. Patients became unable to take their food properly and so they were undernourished. Where squamous metaplasia occurs there are grounds for suspecting increased risk of cancer development.

Abels et al (1942) studied in his patients the pathological changes associated with papillary atrophy of the tongue and oral leukoplakia. The hepatic dysfunction and abnormalities of the gastrointestinal tract were considered to be factors which might contribute to the existence of a dietary deficiency.

Wahi et al (1965) showed that 76.2% of oral cancer patients had subnormal levels of vitamin A. These low serum levels of vitamin A may have been the result of improper nutrition. However, one has to keep in mind the possibility that hypovitaminosis A may be due to the presence of tumour itself. Wahi et al (1958) suggested that dietary deficiency of certain factors may be an important cause of oral cancer.

Previous workers carried out most of their studies in poor countries, and used spectro-analysis and Carr-Price method for vitamin A analysis (Wahi et al 1962; Wahi et al 1965; Ibrahim et al 1977). Most studies suggested that dietary deficiency is associated with oral cancer.

In the present study no clinical evidence of vitamin A deficiency was observed, which might lead to increased risk of oral cancer. Moreover, no differences were found in serum retinol between these groups and normal groups and only 21% of cancer cases showed serum retinol below normal.

In the Crohn's disease group, 57% of patients had serum retinol below normal. There was however, no clinical evidence of vitamin A-deficiency. Dark-adaptation testing was not done here. Full nutritional assessment was not performed, so it is not known if these patients also had protein or energy malnutrition. The serum vitamin E in the Crohn's patients are mainly within the normal range, so fat malabsorption did not occur, suggesting that the low serum retinol is due to protein malnutrition. On the other hand, cancer patients did show significant differences from normal in vitamin E ($p < 0.01$). This has been previously observed in some cases of breast cancer, but the significance is not clear (Wald et al 1984). Main et al (1983) showed a close relationship between vitamin A and protein, and the importance of protein nutrition for transport of serum retinol to its target tissues. They showed that vitamin A-deficiency poses a significant clinical problem in severe Crohn's disease. Our data show low levels of serum retinol, so there is a risk of developing vitamin A deficiency. Vitamin A supplements may be required in these patients. Depending on the degree of small intestinal involvement in Crohn's disease, protein supplements may also be required.

3.3.1.3) Estimation of vitamin A in liver biopsy and serum

Liver biopsies were taken at operation, with the fully informed consent of the patient, from 23 patients aged from 29-83 years undergoing abdominal surgery for various disease states. The protocols were approved by the Hospital Ethical Committee. Eight patients had benign disease and fifteen had neoplastic disease. Biopsies were stored at -20°C until analysis. Total vitamin A as well as retinol ester was analysed. A venous blood sample was withdrawn before operation to estimate retinol in serum. Table 52 shows the concentrations of vitamin A in liver and serum.

There was no significant difference between total control and total cancer patients with respect to liver concentration of total vitamin A, retinol or retinol esters, or in serum retinol (Table 52 and Figures 62-64 respectively)

Considering patients with rectal cancer only, serum retinol was significantly different from controls ($p < 0.05$). There was also a significant differencein serum retinol between control and cancer females ($p < 0.01$) while there was no such difference between males and females in both control and cancer groups for total vitamin A in liver or serum (Table 53).

There was no correlation between age and serum retinol or vitamin A in liver.

There was a poor correlation between total vitamin A in liver and serum retinol in cancer cases ($r = 0.5$) (Figure 65) and in controls ($r = 0.3$) (Figure 66). There was however, better correlation between free retinol in liver and serum retinol in control cases ($r = 0.8$) (Figure 67) , but no similar correlation in cancer cases ($r = 0.3$) (Figure 68).

TABLE 52

CONCENTRATION OF VITAMIN A IN LIVER AND SERUM FROM PATIENTS WITH VARIOUS DISEASES MEDIAN (RANGE)

	Type of disease	No.	Liver Vitamin A (ug/g) Total	Free Retinol	Retinol Palmitate	Other esters	Serum Retinol (µmol/l)
I	BENIGN DISEASE						
	Gall Bladder	4	1047 (800-3900)	106.5 (30-307)	393.5 (350-1404)	552 (410-2188)	3.0 (1.0-4.0)
	Duodenal Ulcer	4	550 (458-550)	91 (35-107)	176 (174-193)	257 (176-291)	1.8 (1.0-3.6)
	Total (control)	8	800 (458-3900)	91 (30-307)	350 (174-1404)	410 (176-2188)	2.2 (1.0-4.0)
II	MALIGNANT DISEASE						
	Stomach Cancer	6	875 (200-2357)	68 (20-230)	276.5 (90-1391)	464 (90-731)	0.9 (0.6-4.4)
	Colorectal cancer	9	778 (88-2363)	45 (10-209)	277 (23-1489)	299 (45-797)	1.6 (0.5-3.2)
	Total (Cancer)	15	778 (88-2363)	60 (10-230)	276 (23-1489)	319 (45-797)	1.2 (0.5-4.4)

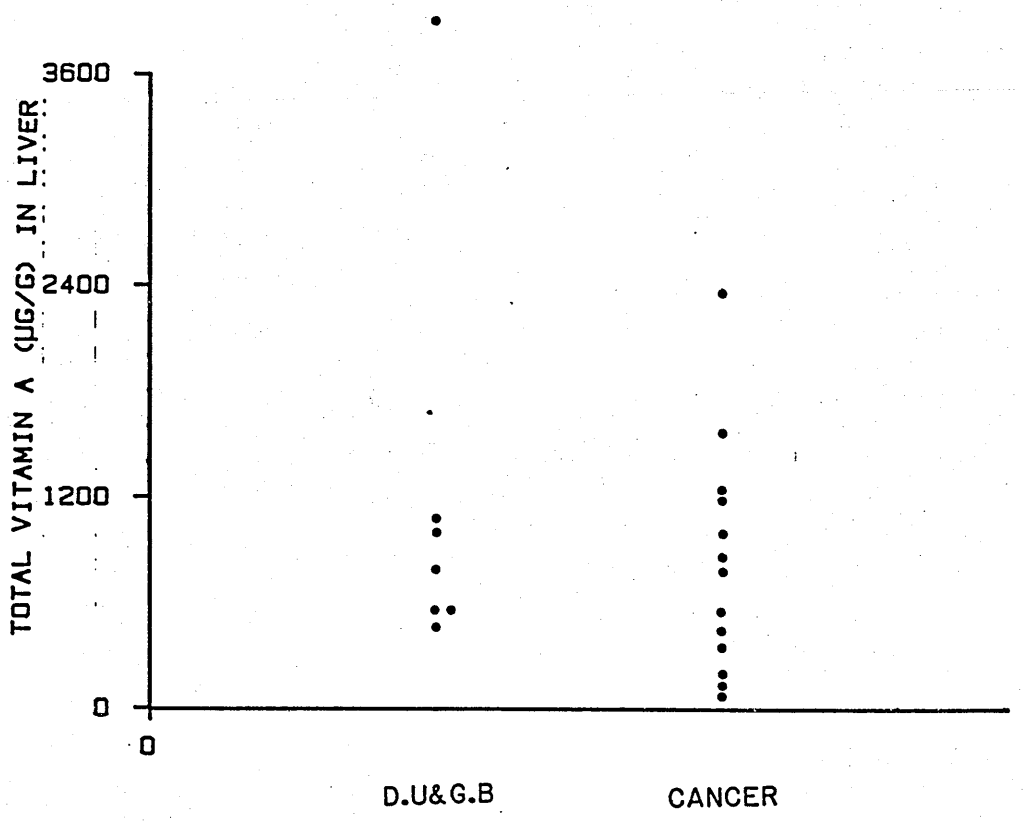


Figure 62: Total vitamin A in liver from patients with benign and malignant disease.

DU = duodenal ulcer
GB = gall bladder disease

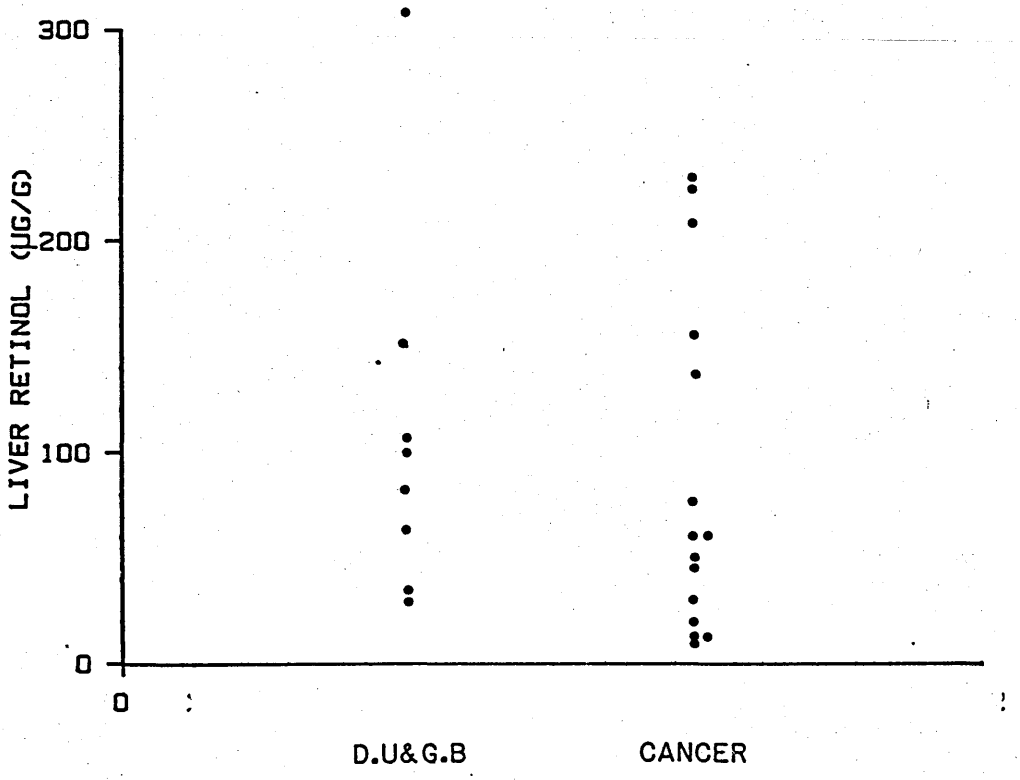


Figure 63: Liver retinol from patients with benign and malignant disease.

DU = duodenal ulcer
GB = gall bladder disease

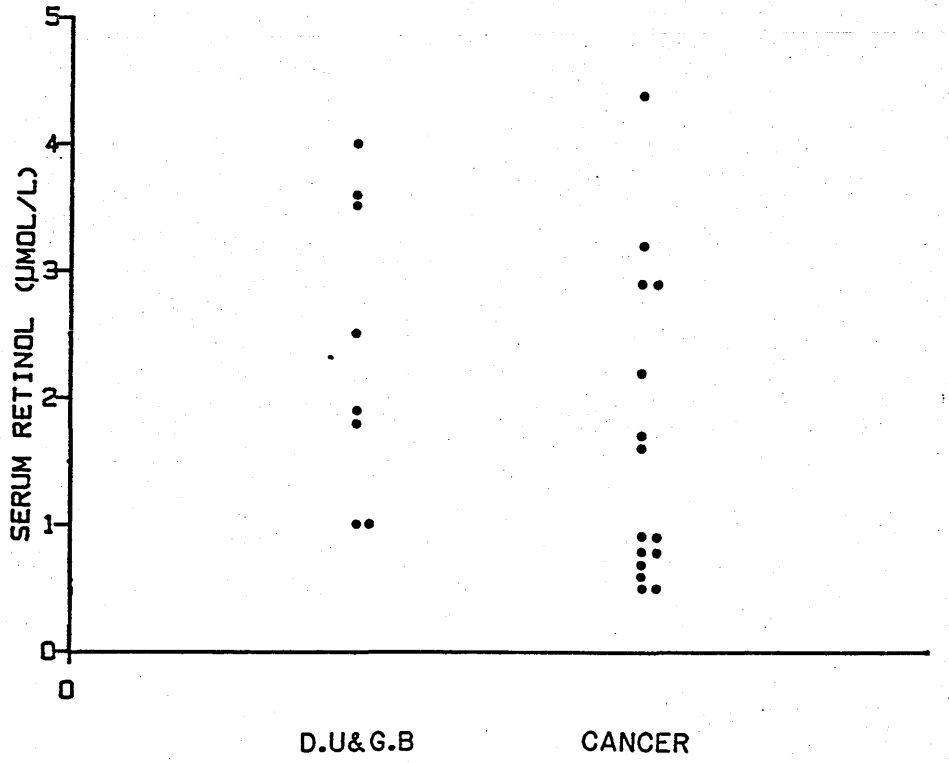


Figure 64: Pre-operative serum retinol from patients with benign and malignant disease

DU = duodenal ulcer
GB = gall bladder disease

TABLE 53

COMPARISON OF LIVER AND SERUM VITAMIN A IN FEMALES AND MALES WITH OR WITHOUT CANCER. MEDIAN (RANGE)

Sex	No.	Total Vitamin A in liver (µg/g)		Serum Retinol (µmol/l)	
		Control (C)	Cancer (Ca)	Control	Cancer
Female	3C	1094	667	3.5	0.9
	6Ca	(800-3900)	(88-2357)	(1.0-4.0)	(0.6-1.6)
		NS	NS	NS	NS
Male	5C	550	860	1.9	1.7
	9Ca	(458-1000)	(125-2363)	(1.0-3.6)	(0.5-4.4)

Ca = cancer
C = control
NS = No signifcance between male and females

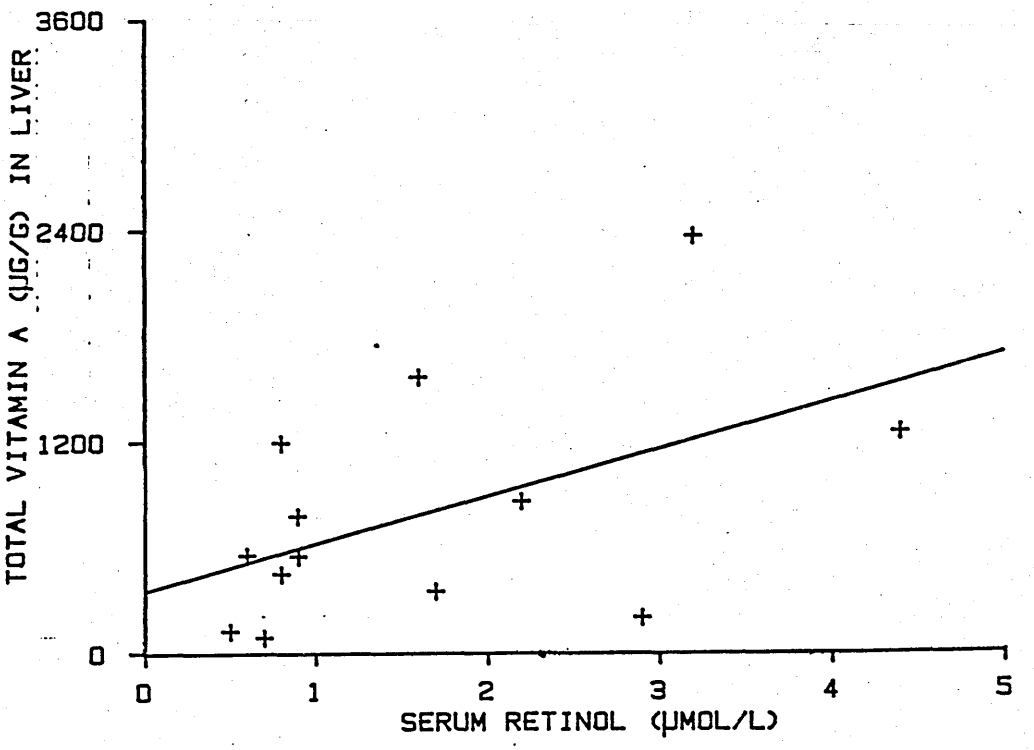


Figure 65: Correlation between serum retinol, and total vitamin A in liver in patients with malignant disease

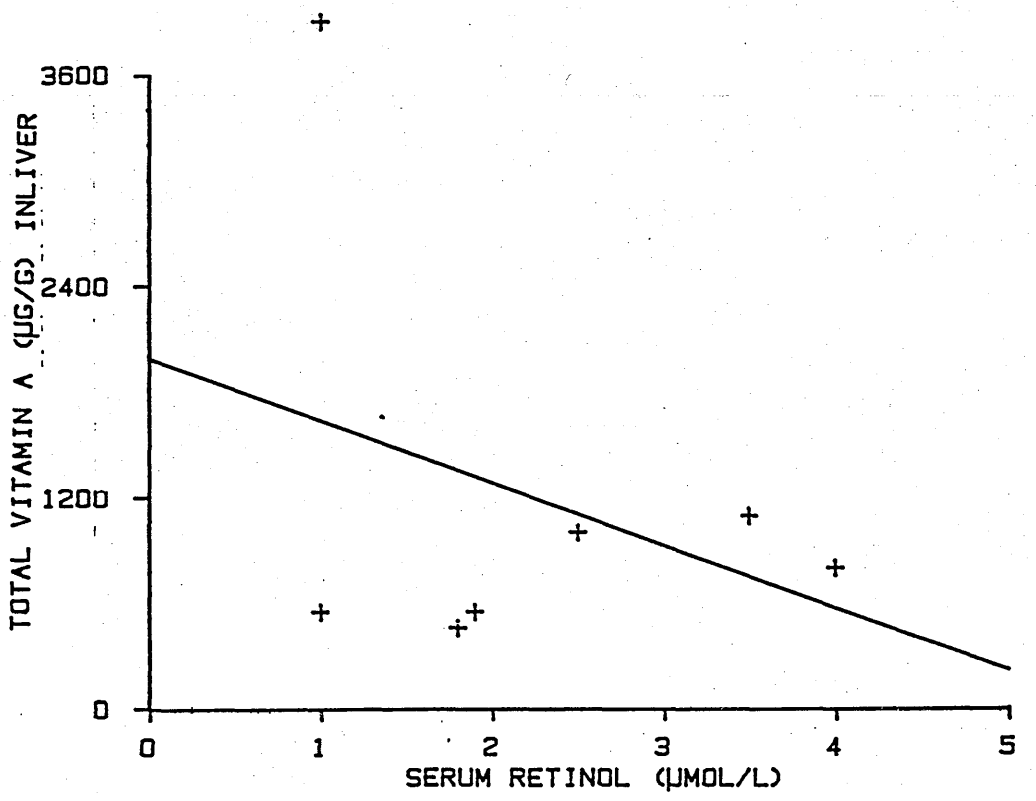


Figure 66: Correlation between serum retinol, and total vitamin A in liver in patients with benign disease.

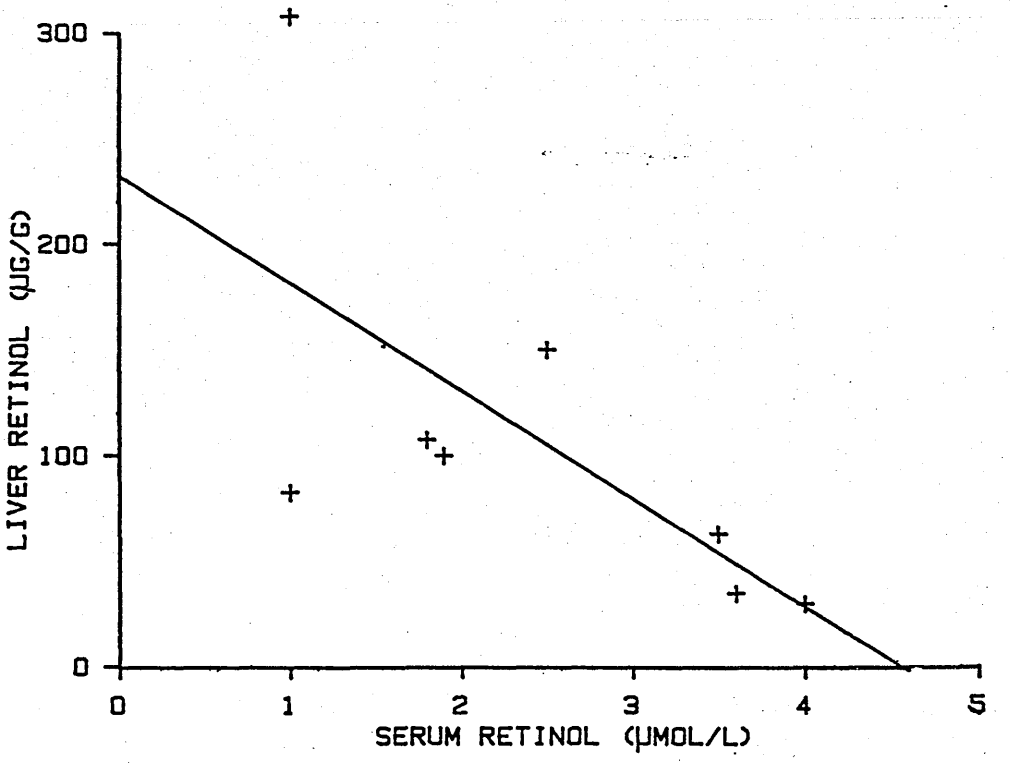


Figure 67: Correlation between serum retinol and liver retinol in patients with benign disease

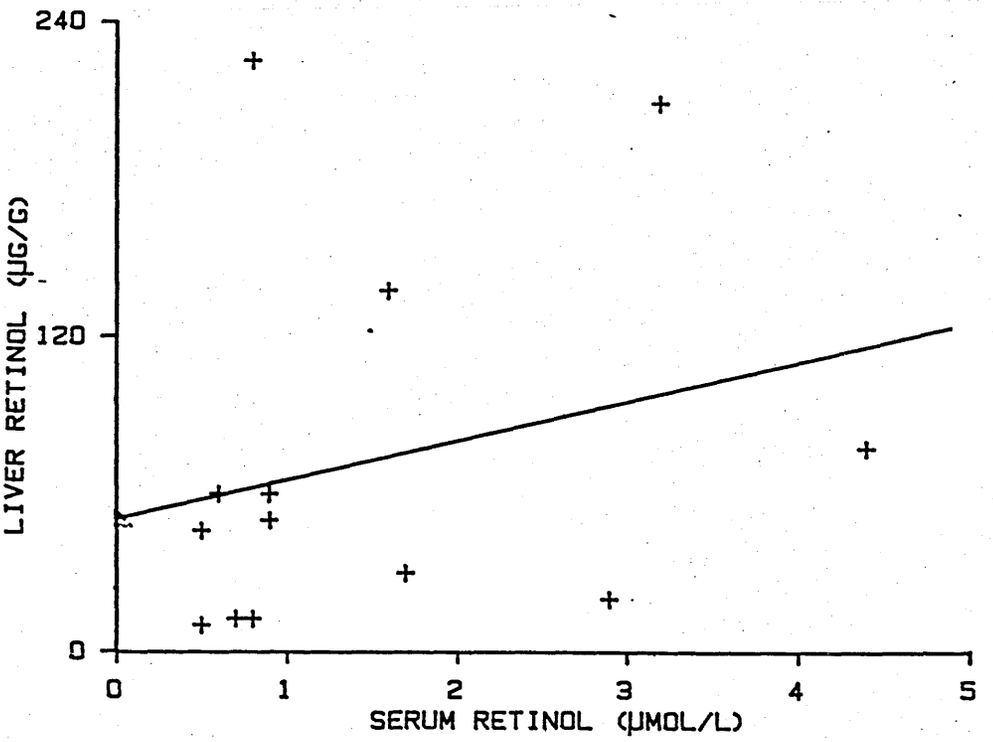


Figure 68: Correlation between serum retinol and liver retinol in patients with malignant disease

Approximately 70% of patients had a liver content of vitamin A around 0-1000µg/g, while 30% had values higher than 1000 µg/g.

Histological examination showed hepatic metastases in some of the cancer patients. Table 54 shows median (range) of serum retinol (µmol/l), total vitamin A and free retinol in liver (µg/g) in patients with and without metastases. Figure 69 shows mean serum retinol (µmol/l) in both metastases and no metastases groups. No significant differences were found

Discussion

Experiments on rats and other animals (Sherman and Boynton, 1925; Kerppola, 1930; Moore, 1931) established that the body's main stores of vitamin A are in the liver. So the hepatic concentration will provide us with an index to the overall vitamin A status. There have been many studies on liver biopsy and post mortem samples (Table 55). A wide variety of vitamin A concentrations have been observed in normal individuals and in different diseases. Under normal conditions of vitamin A sufficiency, 90% or more of the total body reserve of vitamin A is stored in the liver and only 1-3% circulates in the plasma (Moore, 1957; Raica et al 1972). So little correlation would be expected between serum retinol and total liver vitamin A, which we confirmed in this study. However, there was a correlation of $r = 0.8$ between serum retinol and free retinol in liver in normal cases, which may relate to transport of retinol. This correlation was not seen in patients with cancer.

TABLE 54

COMPARISON OF VITAMIN A IN LIVER AND SERUM BETWEEN PATIENTS WITH METASTASES AND WITHOUT METASTASES.

Results are median (range)

Metastases			No Metastases		
Serum Retinol (µmol/l)	Total Vitamin A in liver (µg/g)	Free Retinol in liver (µg/g)	Serum Retinol (µmol/l)	Total Vitamin A in liver (µg/g)	Free Retinol in liver (µg/g)
1.0 (0.5 - 4.0)	1194 (350 - 1563)	107 (30 - 225)	0.9 (0.5 - 3.2)	550 (125 - 2363)	35 (10 - 209)

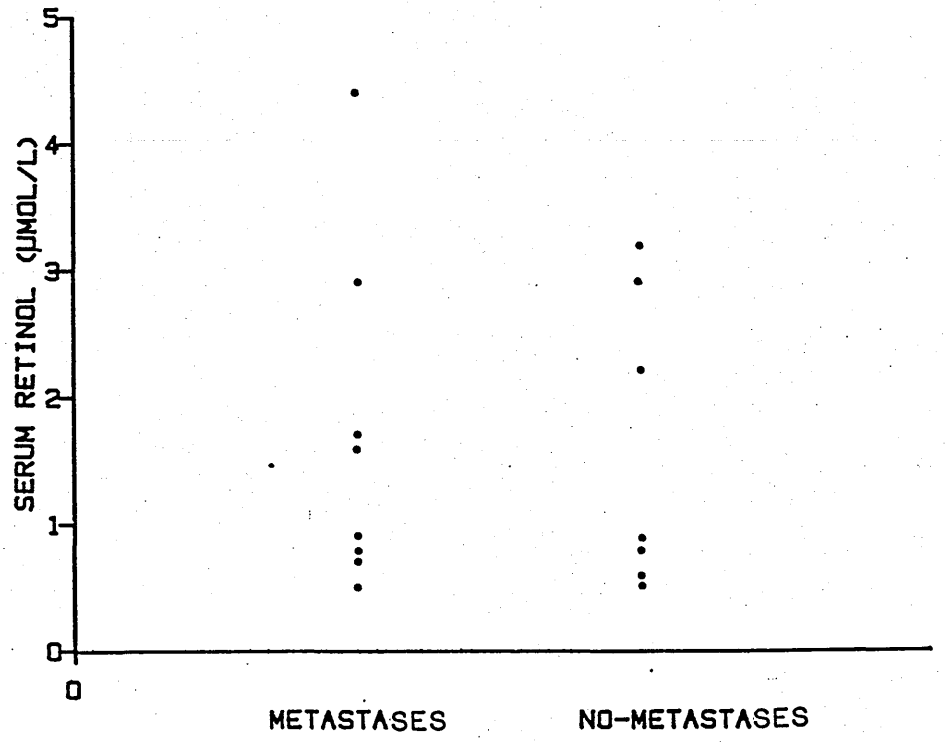


Figure 69: Pre-operative serum retinol in patients with malignant diseases with or without metastases

TABLE 55

COMPARISON BETWEEN VITAMIN A CONTENT IN HUMAN LIVER (μg/g) IN PREVIOUS STUDIES AND IN THE PRESENT STUDY

Author	Liver Vit A (range)	Patients Studied	Author	Liver VitA (range)	Patients Studied
Wolff (1932)	13-48	Newborn and accidental death	Dagadu (1967)	30-1900	Normal values
Ralli et al (1941)	10-19.7	Accidental death	Raica et al (1972)	5-4400	Normal values
	14-489	Chronic alcoholism		2-5500	(dependent on state)
	0-161	Cirrhosis of the liver			
Moore (1957)	42-90	Gastric and duodenal ulcer	Huque and Truswell (1978)	69-1201	Accidental
				10-1132	Cardiovascular disease
	15-75	Gall bladder		6-937	Respiratory disease
	13-96	Cancer		18-563	Cancer
			Olson et al (1979)	0-400	Normal values
Smith and Mathus (1962)	302-333 (3131)	Accidental death (excluded higher values)	Amedee-Manesme et al (1984)	1-400	Normal values
Krause (1965)	3100-4000	Subject taken 15 mg Vitamin A daily	This study	458-3900	Benign diseases
				88-2363	Malignant diseases
Pearson (1967)	100-300	Normal values			

Neither benign surgical disease nor cancer showed any effect on vitamin A levels in liver or on serum retinol. On the other hand, the low values of serum retinol in the pre-operative period found in rectal cancer may be due to liver disease leading to disturbance in mobilization or to protein deficiency. Although most of the rectal cancer patients had metastases, there was no significant difference between serum retinol of patients with metastases compared with non-metastatic cases (Fig 69). This contrasts with the significant difference found in patients with metastases, who underwent colorectal cancer surgery, but whose samples were collected in the early post-operative period (Table 49).

Certain individuals had low levels of vitamin A in liver in our study. This was not necessarily associated with low serum retinol. This is further complicated by the heterogenous distribution of vitamin A in the liver (Amedee-Manesme et al 1984). Similarly, patients with low serum retinol did not always have low liver vitamin A. This could result from impaired protein nutrition, or from an acute-phase reaction to the tumour. Therefore, liver vitamin A measurements are necessary as part of the assessment of vitamin A status.

3.3.2) Studies on animals

3.3.2.1) The effect of iron deficiency and experimental carcinogenesis on vitamin A status in rats

Iron deficiency in common with other nutritional deficiencies, causes widespread and diverse tissue change. In regions of the world where iron deficiency is a serious public health problem there is often a high incidence of oral cancer (Prime et al 1983). Brown-Kelly (1919) and Paterson (1919) were the first to report the association between iron deficiency anaemia and post-cricoid carcinoma. Iron deficiency is reported as causing epithelial atrophy, koilonychia, glossitis and dysphagia. Prime et al (1983) investigated the suggested association between iron deficiency and development of oral squamous-cell carcinoma in the rat. In a subsequent study he investigated whether the influence of iron deficiency in producing earlier tumour in response to carcinogenic application was due to an effect on initiation or promotion. Aspects of their studies will be reported here, in terms of vitamin A status.

Vitamin A is well known for its importance in general growth and differentiation of epithelial tissues, and its deficiency has been shown to lead to metaplastic changes in the epithelia of the respiratory, urogenital and gastrointestinal tracts (Harris et al 1972; Wolback, 1954).

In this study we therefore investigated if there is an association between carcinogenic application, iron deficiency and vitamin A in serum and liver. Specifically, my role in this study was to assist in collection of serum and liver samples from the many different groups of rats, and to perform all vitamin A analyses.

In Study I Prime et al (1983) investigated the effect of iron deficiency on oral carcinogenesis by using 30 young adult male Charles River white rats. These rats aged 6-8 weeks were housed in polyethylene cages 2 or 3 per cage. They were divided into 2 groups, iron sufficient (Group 1) and iron deficient (Group 2). In group 1, 15 rats were fed a specially-prepared powdered diet with an iron concentration of approximately 140 mg/kg of diet (the iron used was in the form of ferrous sulphate). Group 2, 15 rats received a similar diet with iron concentration 12 mg/kg (National Academy of Science, 1978, indicated that 35 mg/kg iron is necessary for growth). The diet was prepared taking account of the other nutrients, carbohydrate, protein, fats and vitamins. All animals received 15 g of diet daily and had glass distilled water ad-lib. Blood samples were collected from control animals in group 1 on 6 occasions during the course of the experiment in order to monitor the iron status. It had been hoped that iron deficiency anaemia could be induced in group 2 animals by dietary means alone, but after 8 weeks of iron deficient diet the animals showed an average fall of only 1.56 g/dl of haemoglobin. A regime of repeated bleeding was instituted both to induce and to maintain further iron deficiency. In group 2, animals were bled at 2-3 week intervals, but towards the end of the experiment this was extended to 3-4 weeks. In total the iron deficient animals were bled on 13 occasions throughout the course of the experiment.

The carcinogen application technique was that described by Wallenius and Lekholm (1973). All animals were treated with 0.5% 4-Nitroquinoline-N-oxide in propylene glycol which was

painted on the palate 3 times weekly. After the application of carcinogen, the animals were followed up till sacrifice. The oral cavity of each rat was examined weekly. The results in iron deficient and iron sufficient rats with and without carcinogen were compared.

In Study I all animals (Groups 1 and 2) were painted with 4NQO for most of the period of study while in study II 60 animals were used and painted with 4 NQO for 8 weeks (Groups 3 and 4) or 14 weeks (Groups 5 and 6) and were then monitored until 40 weeks, when they were sacrificed. The protocol of the two studies are summarised in Table 56.

In Study I blood and liver samples were taken at sacrifice, while in Study II, blood was taken at the start of the study, 1-2 weeks after 8 or 14 weeks of carcinogen application, and at sacrifice. Liver was collected at the end of the experiment (sacrifice). The effect of iron status, weight and of carcinogen on vitamin A status was studied.

Results

In Study I, the mean haemoglobin values of group 1 did not change significantly from a starting value of 14.1 g/dl to 13.8 g/dl at the sacrifice. In group 2 the mean haemoglobin values at the start of the diet was not significantly different from that of group 1. However, the mean haemoglobin of the animals fell steadily until at the time of the first carcinogen application it was 10.1 g/dl and by sacrifice it had reached 8.2 g/dl and was significantly different from group 2 ($p < 0.001$) at the time of first carcinogen application and at sacrifice. Both groups developed similar squamous cell carcinomas, but tumour

TABLE 56

IRON STATUS, TIME PERIODS OF APPLICATION OF CARCINOGENESIS IN STUDY I AND II

	Group	No. of rats	Iron status	Carcinogen start	Application of carcinogen	Sacrificed
Study I	1	15	+ Fe	after 5 weeks	most of period	35 weeks
	2	15	- Fe	after 10 weeks	most of period	35 weeks
Study II	3	15	+ Fe	6-10 weeks	8 weeks	40 weeks
	4	15	- Fe	6-10 weeks	8 weeks	40 weeks
	5	15	+ Fe	6-10 weeks	14 weeks	40 weeks
	6	15	- Fe	6-10 weeks	14 weeks	40 weeks

development was significantly earlier in iron deficient groups mean 183 day (85-224) compared with control (iron sufficient group) mean 229 days (174-257) with $p < 0.02$). Iron deficient animals showed a significantly greater incidence of tongue tumours ($p < 0.01$) and iron-sufficient animals (control) showed a significantly greater incidence of palatal tumours ($p < 0.01$) (Table 57).

The mean weights of animals in the iron-sufficient and iron-deficient rats throughout the experiment are shown in Table 58.

No significant difference in weights were found between the two groups at the start of diet, start of carcinogen application or at sacrifice.

Retinol in serum was measured at sacrifice only and all rats showed a very low serum concentration. There was no significant difference between iron sufficient and iron deficient rats in terms of retinol and sacrifice (Table 59).

Liver retinol and retinol palmitate were undetectable at the time of sacrifice.

In Study II, the mean haemoglobin value of the iron sufficient group 3 showed a change from 11.2 g/dl at the start to 12.3 g/dl at sacrifice. In the iron deficient group 4, the mean haemoglobin values showed a change from 11.1 g/dl at the start of the study to 7.2 g/dl, at sacrifice with a significant difference ($p < 0.001$) between the two groups at 1-2 weeks after 8 weeks carcinogen application and sacrifice (Table 60).

TABLE 57

COMPARISON OF TUMOUR DEVELOPMENT IN GROUP 1 AND 2

Group	Iron Status	No. of animals showing tumour	Mean days (range) to tumour development after Ca-application	No. of animals with palatal atypia	No. of animals with tongue atypia	No. of animals with palatal and tongue atypia
1	+ Fe	11/15	229 (174 - 257)	5	1	5
2	- Fe	8/15	183 (85 - 224)	1	6	1
Significance		NS	0.02	0.01	0.01	-

From Prime et al (1983)

TABLE 58

WEIGHT (gm) OF ANIMALS IN GROUPS 1 and 2 (MEAN AND RANGE)

Group	Iron Status	Start of diet	Start of carcinogen	Sacrifice
1	+Fe	173 (130-250)	226.7(225-340)	274.3 (150-370)
2	-Fe	186 (110-250)	226.0(225-315)	347.3 (155-340)

from Prime et al (1983)

TABLE 59

SERUM RETINOL IN GROUPS 1 AND 2 AT SACRIFICE (MEAN)

Group	Iron status	No. of rats	Serum retinol at sacrifice (µmol/l)
1	+Fe	9	0.1 (4 animals) <0.1 (5 animals)
2	-Fe	9	0.1 (6 animals) <0.1 (3 animals)

TABLE 60

HAEMOGLOBIN (gm/dl) IN GROUPS 3 and 4 (MEAN ± SEM)

Group	No. of Rats	Iron Status	Start of diet	1-2 weeks post carcinogen application	Sacrifice
3	15	+ Fe	11.2 ± 0.2	14.6 ± 0.2	11.9 ± 0.09
4	15	- Fe	11.1 ± 0.2	8.6 ± 0.4	6.7 ± 0.3
Significance (Groups 3 & 4)			NS	$p<0.001$	$p<0.001$

There was no significant difference in the weights of iron-sufficient and iron-deficient animalsat all time points (Table 61). Mean serum retinol in both groups fell approximately 1.3 µmol/l by 1-2 weeks after 8 week carcinogen application from the start of the experiment and continued to fall to very low values at the time of sacrifice (Table 62). There was no significant difference in serum retinol between the iron-sufficient and deficient group at any time.

Figure 70 shows the change in weight between the start of experiment and 1-2 weeks post carcinogen application plotted against serum retinol in the iron-sufficient group. The correlation was $r = -0.8$. There was also a correlation in both groups between the change in serum retinol and change in haemoglobin from the start of the study to the end of carcinogen, between end of carcinogen and time of sacrifice, and between start of study and time of sacrifice and these correlations were greater for iron-deficient rats (Table 63 and Figures 71 and 72).

The mean haemoglobin in iron sufficient group 5 showed a minimal change from 13.1 g/dl at the start of study to 12.9 g/dl at sacrifice. In iron-deficient group 6 the mean haemoglobin values showed a change from 13.9 g/dl at the start of the study to 8.0 g/dl at sacrifice with significant difference of $p < 0.001$ between the two groups at 1-2 weeks post 14 weeks of carcinogen application and at sacrifice (Table 64).

The iron sufficient group 5, was heavier than the iron deficient group 6, both after 14 weeks of carcinogen application and at sacrifice (Table 65, Figure 76).

TABLE 61

WEIGHT (gm) FOR GROUPS 3 and 4 AT DIFFERENT TIMES (MEAN ± SEM)

Group	No. of rats	Iron status	Start of diet	1-2 weeks post carcinogen application	Sacrifice
3	15	+ Fe	256 ± 18.3	304 ± 11.6	411 ± 25.8
4	15	- Fe	281 ± 13.7	317 ± 8.0	367 ± 15.7
Significance (Group 3 & 4)			NS	NS	NS

TABLE 62

SERUM RETINOL (μmol/1) FOR GROUPS 3 and 4 AT DIFFERENT TIMES (MEAN ± SEM)

Group	No. of rats	Iron Status	Start of diet	1-2 weeks post carcinogen application	Sacrifice
3	15	+ Fe	2.4 ± 0.1	1.1 ± 0.1	0.16 ± 0
4	15	- Fe	2.5 ± 0.1	1.2 ± 0.03	0.17 ± 0
Significance (Group 3 & 4)			NS	NS	NS

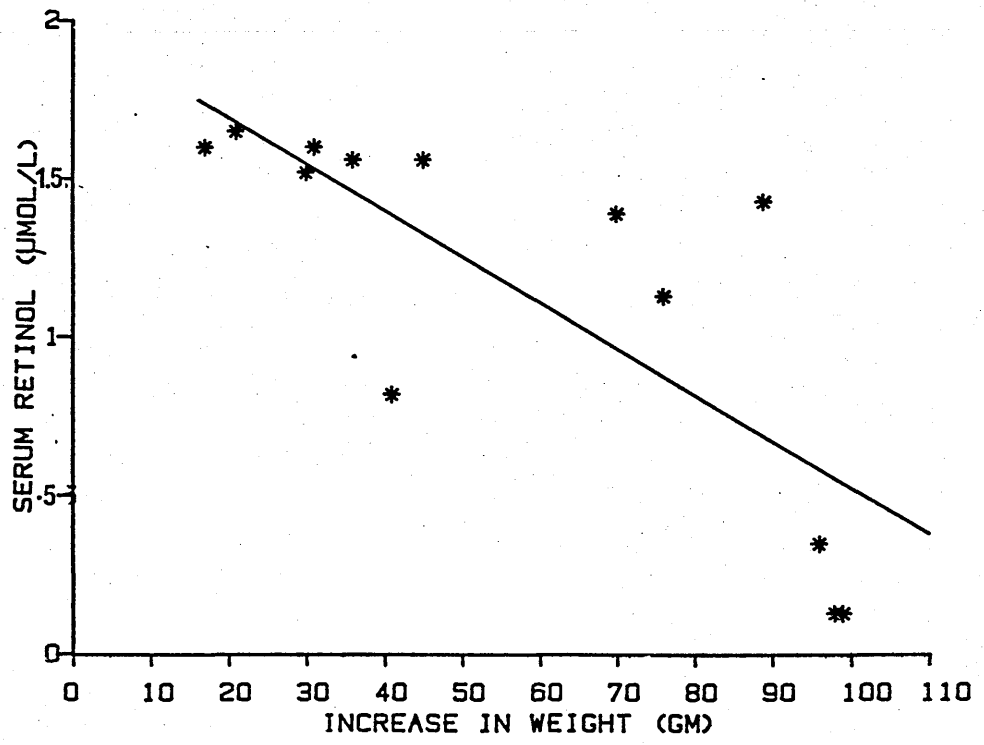


Figure 70: Correlation between serum retinol and change in weight. A blood sample was taken at start, and 1-2 weeks post carcinogen in iron-sufficient rats treated for 8 weeks, and the change in weight was noted.

TABLE 63

CORRELATION BETWEEN SERUM RETINOL, WEIGHT, AND HAEMOGLOBIN IN RATS AT DIFFERENT STAGES

Correlation between Stages	Group No.	Correlation of change of Serum retinol and change of weight		Correlation of change of serum retinol and change in haemoglobin		Correlation of change of weight and change of haemoglobin	
		Iron-sufficient (+Fe)	Iron-deficient (-Fe)	Iron-sufficient (+Fe)	Iron-deficient (-Fe)	Iron-sufficient (+Fe)	Iron-deficient (-Fe)
Start of study against 1-2 weeks post-carcinogenesis	3&4	r =-0.8	r = 0.4	r = 0.2	r = 0.5	r = 0.3	r = 0.3
	5&6	r = 0.1	-	r = 0.1	-	r = 0.4	r = -0.4
1-2 weeks post carcinogenesis against sacrifice	3&4	r = 0.1	r = 0.2	r = 0.5	r = 0.9	r = 0.1	r = 0.3
	5&6	r = -0.4	-	r = 0.7	-	r = 0.1	r = 0.2
Start of study against sacrifice	3&4	r = 0.4	r = 0.5	r = 0.3	r = 0.6	r = 0.3	r = 0.6
	5&6	r = -0.5	r = 0.04	r = 0.1	r = 0.6	r = 0.3	r = -0.02

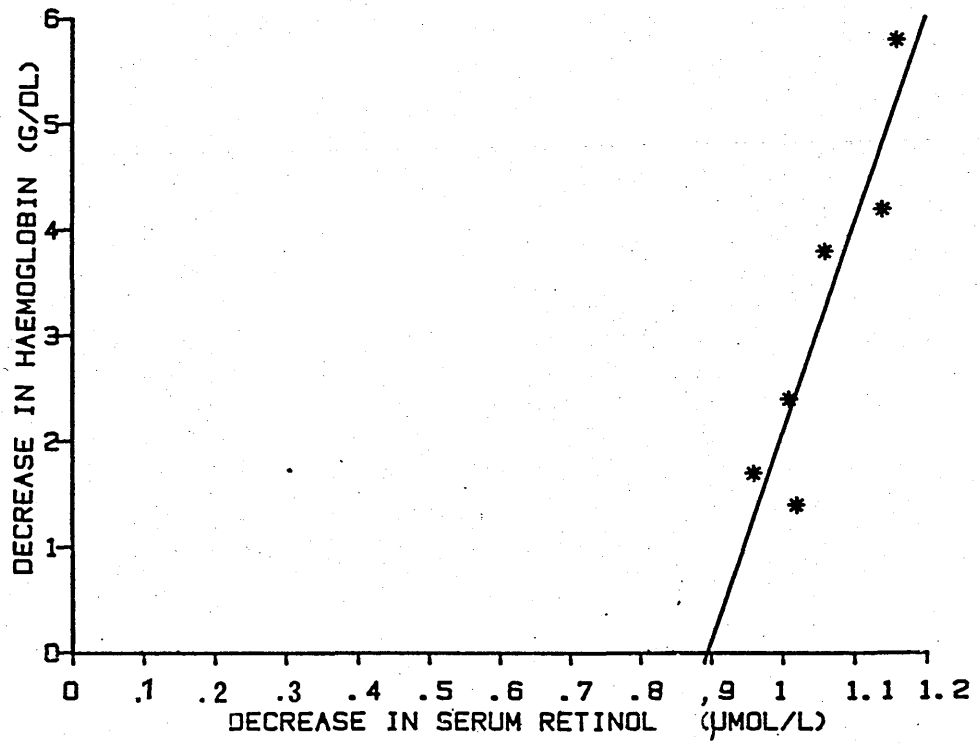


Figure 71: Correlation between decrease in serum retinol and haemoglobin. Blood samples were taken 1-2 weeks post carcinogen and at sacrifice in iron-deficient rats treated with carcinogen for 8 weeks.

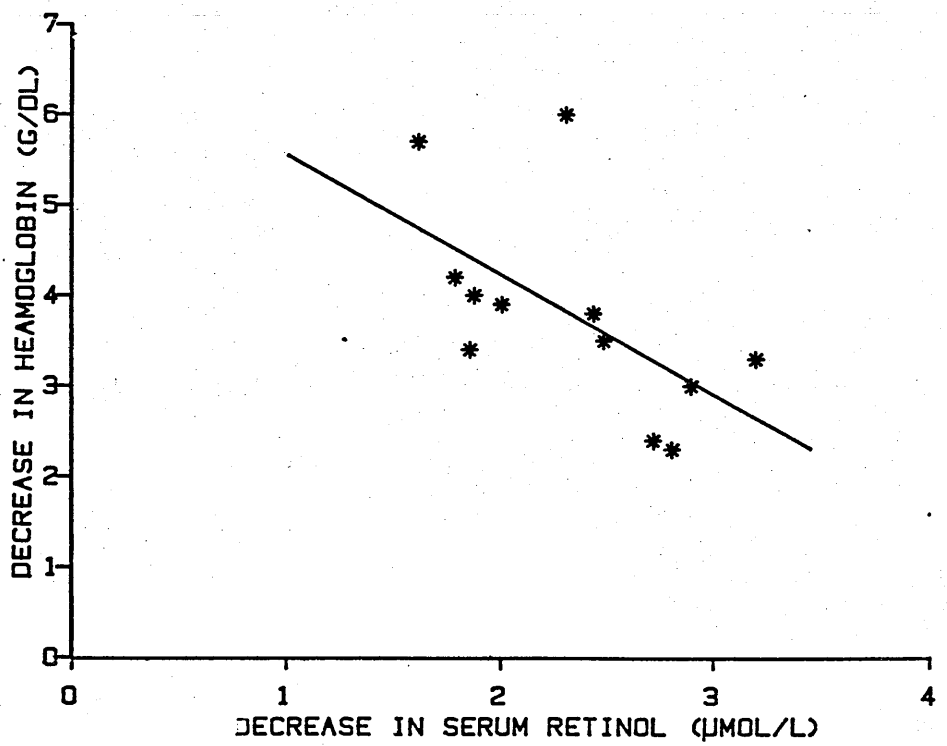


Figure 72: Correlation between decrease in serum retinol and in haemoglobin. Blood samples were taken from iron-deficient rats at start and at sacrifice (40 weeks) having been treated with carcinogen for 8 weeks.

TABLE 64

HAEMOGLOBIN (gm/dL) FOR GROUPS 5 and 6 AT DIFFERENT TIMES (MEAN ± SEM)

Group	No. of rats	Iron Status	Start of diet	1-2 weeks post carcinogen application	Sacrifice
5	15	+ Fe	13.1 ± 0.2	13.8 ± 0.3	12.6 ± 0.3
6	15	- Fe	13.9 ± 0.3	8.8 ± 0.5	7.6 ± 0.4
Significance (Group 5 & 6)			NS	$p < 0.001$	$p < 0.001$

TABLE 65

WEIGHT (gm) FOR GROUPS 5 and 6 AT DIFFERENT TIMES (MEAN ± SEM)

Group	No. of rats	Iron Status	Start of diet	1-2 weeks post carcinogen application	Sacrifice
5	15	+ Fe	218 ± 10.1	334 ± 6.5	364 ± 10.1
6	15	- Fe	200 ± 7.0	265 ± 7.0	313 ± 11.4
Significance (Group 5 & 6)			NS	$p < 0.001$	$p < 0.001$

The mean serum retinol dropped during carcinogen application from the start of the study (Table 66). Carcinogen application for 14 weeks in iron sufficient rats led to a fall in serum retinol from 2.4 ± 1.0 µmol/l to 0.3 ± 0.4 µmol/l, which was significantly lower than in iron sufficient rats of 8 weeks application 2.4 ± 0.4 µmol/l, 1.1 ± 0.5 µmol/l. The fall continued and reached very low values at sacrifice in both iron sufficient (0.16 µmol/l, 0.25 µmol/l) and iron deficient (0.17 µmol/l, 0.18 µmol/l) groups with no significant difference between the two groups at any time.

There was a significant correlation in the change in haemoglobin between 1-2 weeks after 14 weeks of carcinogen application and sacrifice plotted against the change in serum retinol in the iron sufficient group ($r = 0.7$) but these changes were very small (Figure 73) Iron deficient animals showed a correlation between the changes in haemoglobin between start of the study and sacrifice plotted against the change of serum retinol ($r = 0.6$) (Figure 74).

Figures 75 and 76 show the weight of the two groups (3 and 4; 5 and 6) at 1-2 weeks after 8 or 14 weeks of carcinogen application respectively and at sacrifice. There is a significant difference between iron sufficient and iron deficient groups 5 and 6 ($p < 0.001$) but there was no difference between iron-sufficient and iron deficient groups 3 and 4 at either of these time points. In group 4 the iron deficient rats were significantly heavier at sacrifice than group 6 ($p < 0.01$) but no such difference was found between iron sufficient groups 3 and 5.

TABLE 66

SERUM RETINOL (μmol/l) FOR GROUPS 5 AND 6 AT DIFFERENT TIMES (MEAN ± SEM)

Group	No. of rats	Iron status	Start of diet	1-2 weeks post carcinogen application	Sacrifice
5	15	+ Fe	2.4 ± 0.3	0.3 ± 0.1	0.25 ± 0
6	15	- Fe	1.8 ± 0.2	-	0.18 ± 0
Significance (Group 5 & 6)			NS	-	NS

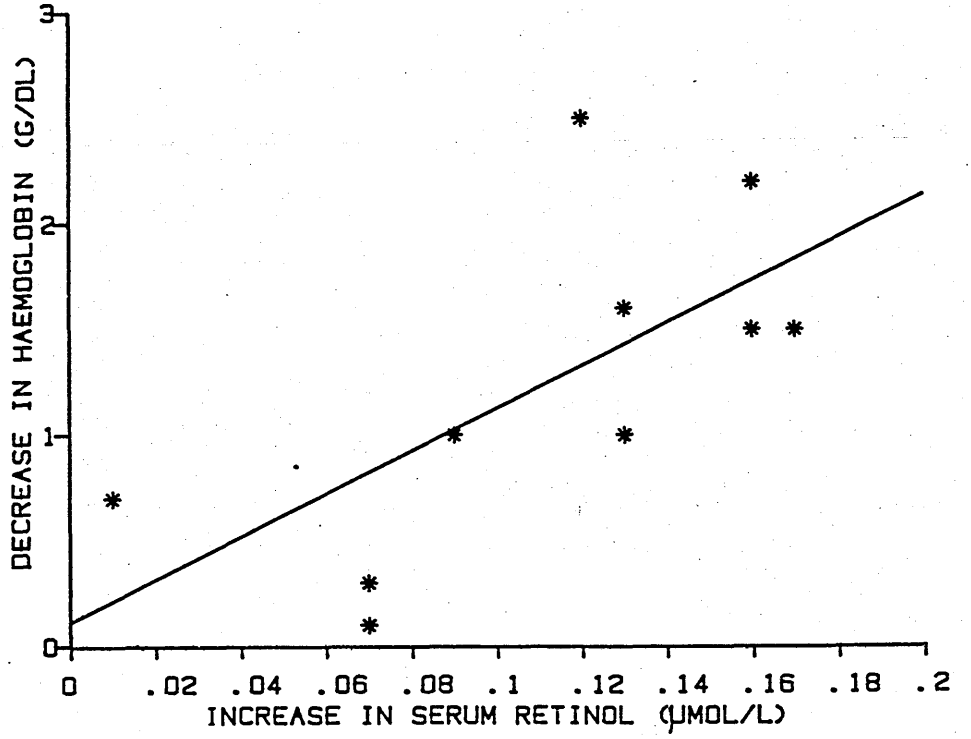


Figure 73: Correlation between increase in serum retinol and decrease in haemoglobin. Blood samples were taken from iron-sufficient rats at 1-2 weeks post carcinogen and at sacrifice (40 weeks) having been treated with carcinogen for 14 weeks.

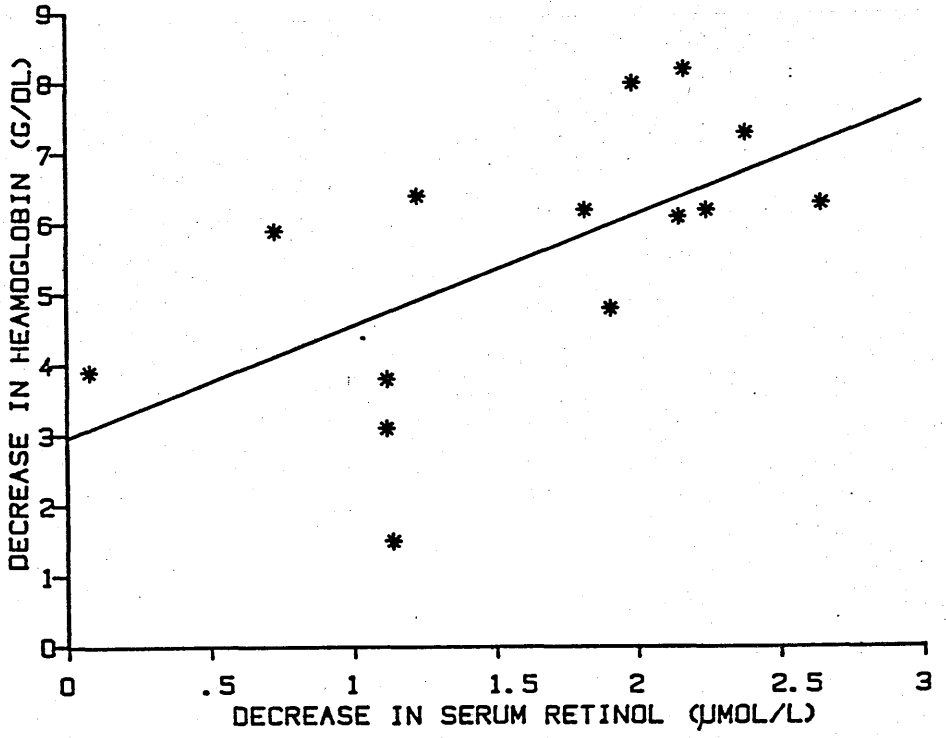


Figure 74: Correlation between decrease in serum retinol and decrease in haemoglobin concentration. Blood samples were taken from iron-deficient rats, at start and at sacrifice (40 weeks) having been treated with carcinogen for 14 weeks.

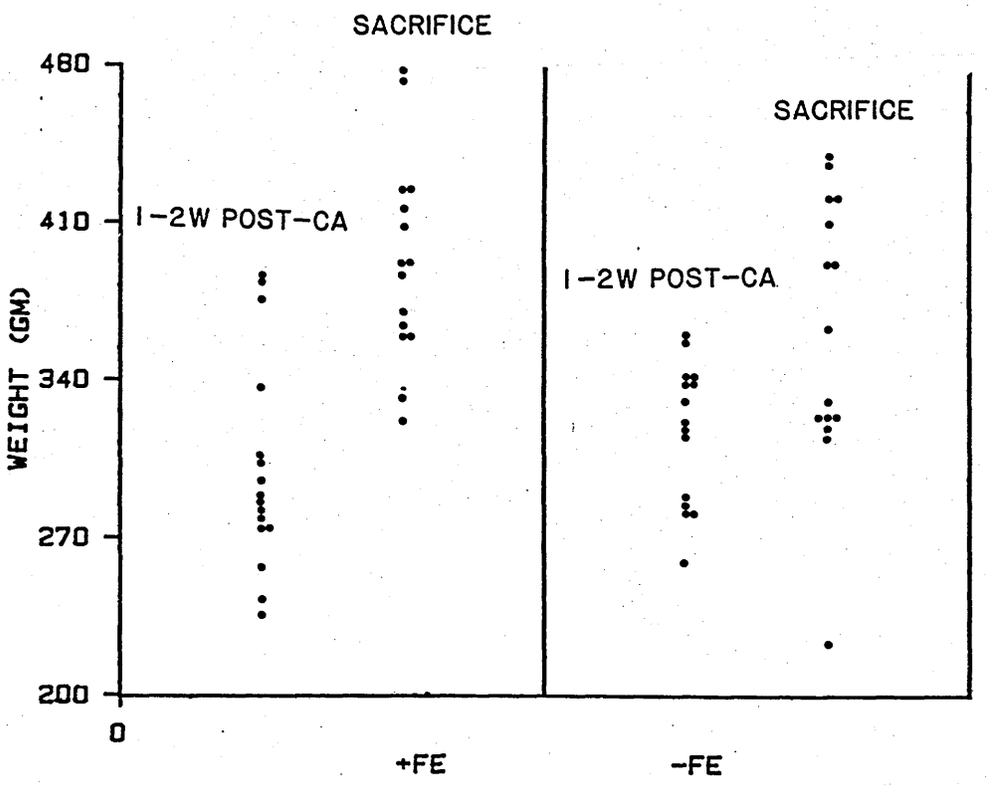


Figure 75: Weight of iron-sufficient, and iron-deficient animals at 1-2 weeks post carcinogen and at sacrifice (40 weeks), having been treated with carcinogen for 8 weeks.

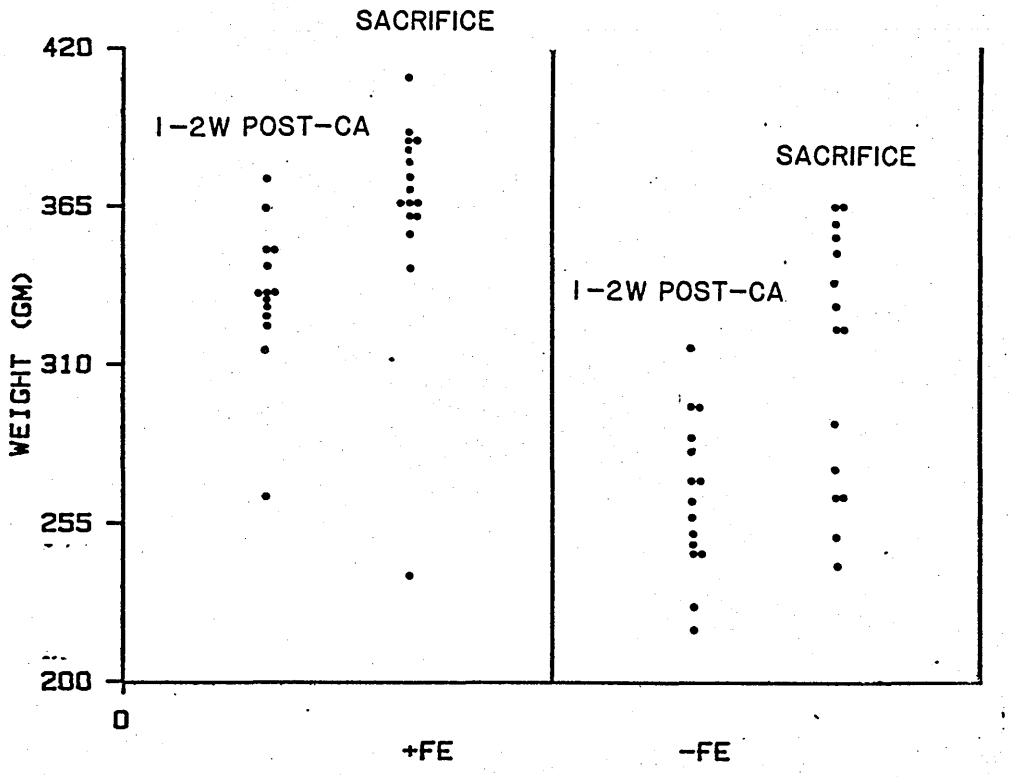


Figure 76: Weight of iron-sufficient, and iron-deficient animals at 1-2 weeks post-carcinogen and at sacrifice (40 weeks) having been treated with carcinogen for 14 weeks.

Figures 77-79 show the mean weight, serum retinol and haemoglobin values of all the experimental animals at the start of the study, end of 8 or 14 weeks carcinogen application and at the time of sacrifice respectively.

No detectable vitamin A could be found at sacrifice in the liver of all animals treated with carcinogen. The values of vitamin A in liver stored for one year of control male animals fed on normal food at the same time as the experimental animals are shown in Table 67, in comparison with fresh liver from male rats. The results indicate that after one year of storage most of the retinol palmitate has been converted to other retinol esters or to free retinol. It is however readily detected by saponification and measurement of total retinol.

In Study II, the scores which are an arithmetic figure to produce the degree of dysplasia, show that palate atypia scores in iron-deficiency animals were significantly higher than the tongue atypia scores (less severe stage) in each group ($p < 0.01$). Both atypia represent pre-malignant stages. The tongues of most animals showed absent or minimal dysplasia and no differences were found between the groups.

The palates of animals painted for 14 weeks showed significantly higher atypia scores than animals painted for 8 weeks in both iron-sufficient and iron-deficient groups ($p < 0.003$). There was no significant difference if comparison was made between the palatal atypia scores of the iron-deficient animals with the corresponding iron-sufficient animals after either 8 weeks or 14 weeks of carcinogen application (Table 68).

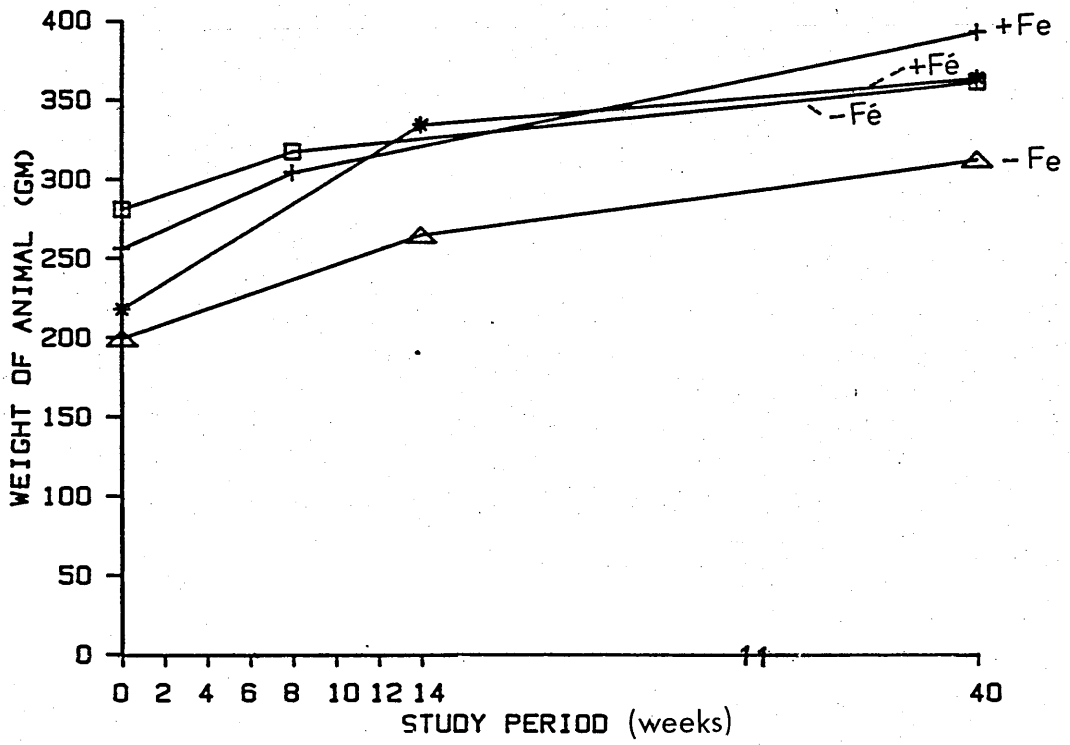


Figure 77: Mean weight of animals in each group during the study period.

□ iron-deficient, 8 week carcinogen
\+ iron-sufficient, 8 week carcinogen
Δ iron-deficient, 14 week carcinogen
* iron- sufficient, 14 week carcinogen

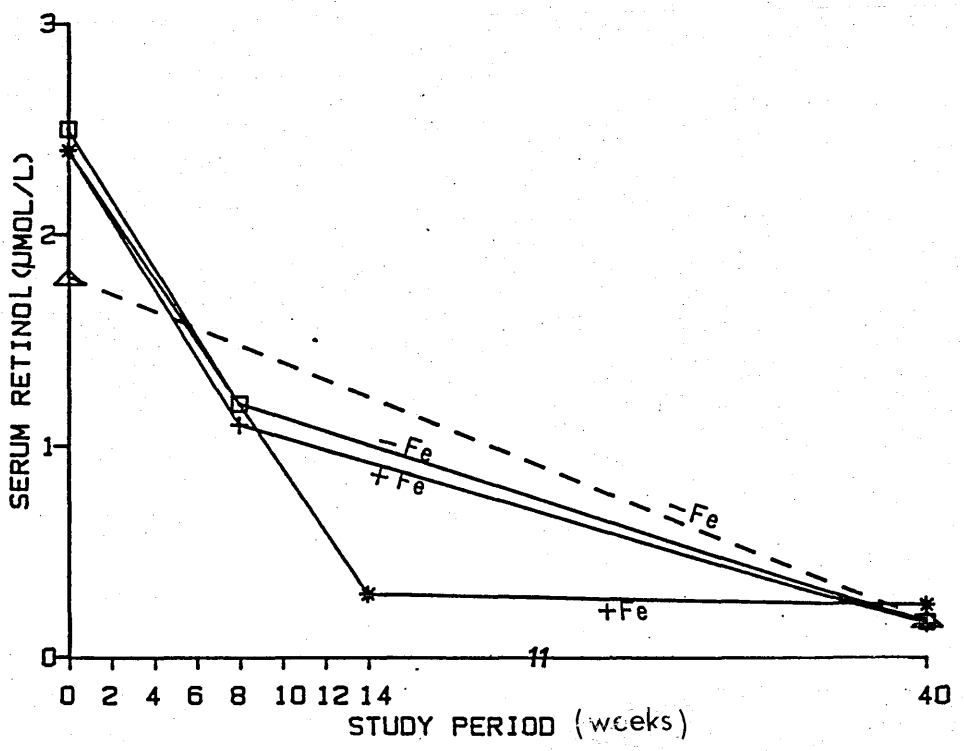


Figure 78: Mean serum retinol in each group during the study period.

□ iron-deficient, 8 weeks carcinogen
\+ iron-sufficient, 8 weeks carcinogen
Δ iron-deficient, 14 weeks carcinogen
* iron-sufficient, 14 weeks carcinogen

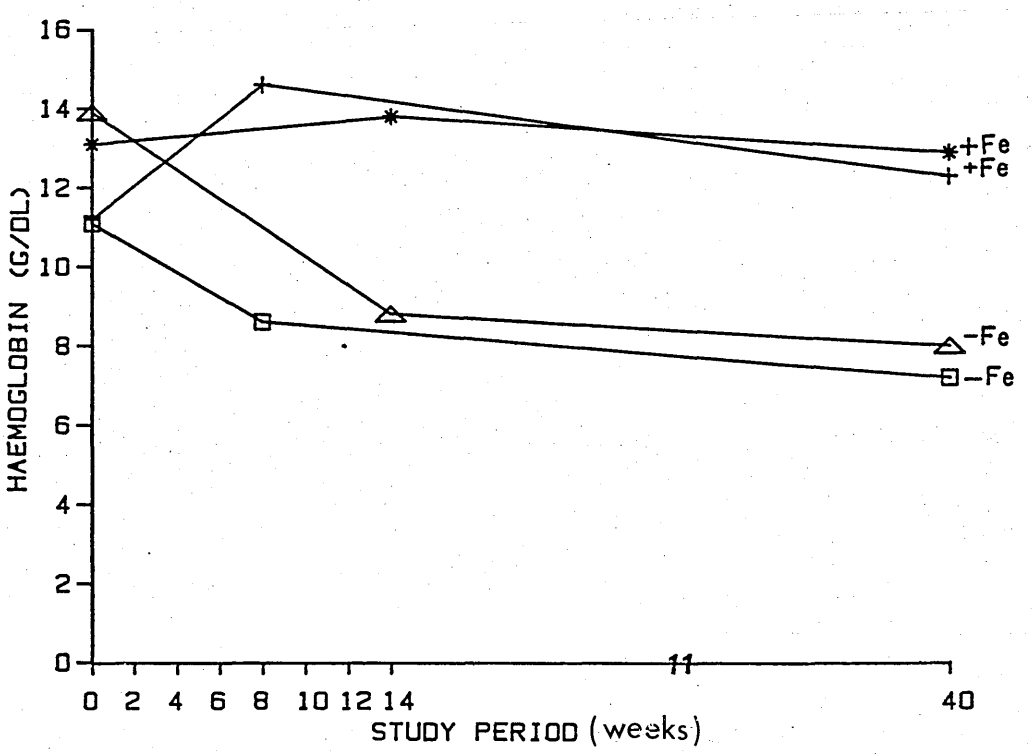


Figure 79: Mean blood haemoglobin of animals in each group during the study period

□ iron-deficient, 8 weeks carcinogen
\+ iron-sufficient, 8 weeks carcinogen
Δ iron-deficient, 14 weeks carcinogen
* iron-sufficient, 14 weeks carcinogen

TABLE 67

COMPARISON BETWEEN VITAMIN A CONTENT IN LIVER STORED FOR 1 YEAR AND FRESH LIVER FROM NORMAL RATS

Number	One year storage				Fresh			
	Total[1] (µg/g)	Retinol[2] (µg/g)	Retinol palmitate[3] (µg/g)	Retinol[4]	Total[1] (µg/g)	Retinol[2] (µg/g)	Retinol palmitate[3] (µg/g)	Retinol[4]
1	500	21	217	118	150	9.5	223	122
2	1400	116	230	126	250	10.0	270	147
3	300	27	96	53	300	5.5	232	127
4	1200	123	112	61	150	9.0	180	98
5	1550	398	162	89	150	9.0	204	112
6	2850	218	135	74				
mean ($\bar{x}$)	1300	150.5	158.7	86.8	200	8.6	221.8	121.2
%		11.6	86.8	6.7		4.3		61

1 Total retinol and saponification followed by HPLC measurement
2 Free retinol - HPLC analysis
3 Retinol palmitate - HPLC analysis
4 Calculated amount of retinol in retinol palmitate

TABLE 68

EPITHELIAL ATYPIA SCORES ASSESSED BY SMITH AND PINDBURG (1969) TECHNIQUE

Animal	Group 1 - Iron-deficient				Group 2 - Iron-sufficient			
	8 weeks NQO		14 weeks NQO		8 weeks NQO		14 weeks NQO	
	Palate	Tongue	Palate	Tongue	Palate	Tongue	Palate	Tongue
1	4	3	20	0	5	3	11	0
2	4	0	35	0	3	3	12	2
3	4	0	24	2	8	2	27	2
4	6	3	8	3	29	15	28	1
5	3	0	14	2	2	1	26	23
6	1	0	34	0	5	1	1	2
7	32	5	24	3	3	1	34	31
8	8	3	30	5	3	3	5	8
9	3	1	26	2	7	1	22	0
10	5	0	40	3	3	1	44	3
11	14	0	44	13	3	0	9	1
12	29	3	28	1	6	2	24	2
13	12	6	18	4	20	0	48	0
14	1	1	40	7	23	0	35	0
15	28	2	48	4	6	0	32	3

From Prime et al (1986)

Table 69 shows a correlation between epithelial atypia scores and serum retinol in both groups. The only correlation found is between scoring in tongue and serum retinol 1-2 weeks post carcinogenesis in iron-deficiency rats painted for 8 weeks.

Groups of iron-sufficient and iron-deficient male rats without carcinogen were study as control. There was no significant difference between the weight of animals or serum retinol in the two groups or in comparison with control rats fed on a normal diet. Table 70 shows the weight, serum retinol levels and haemoglobin for these rats.

Discussion

In both studies, carcinogen had no effect on weight and the animals gained weight from the start of the experiment until sacrifice. There was no difference between the weight at the end of the experiment compared with those of normal rats. Figure 70 shows an inverse correlation between the increase in weight and serum retinol ($r = -0.8$), in iron sufficient groups after 8 weeks of carcinogen application. This suggests that the more rapid the rate of growth, the greater the fall in serum retinol. After 14 weeks this correlation was not observed ($r = 0.1$). This probably results from the low plasma retinol seen in all animals after 14 weeks.

On the other hand, serum retinol dropped to very low values at the time of sacrifice, and the animals show a remarkable change from the starting point to 1-2 weeks post application period. The animals who were treated for 14 weeks show a lower value of serum retinol than those treated for 8 weeks. Carcinogen had no effect on weight of animals in both

TABLE 69

CORRELATION BETWEEN EPITHELIAL ATYPIA SCORING AND SERUM RETINOL 1-2 WEEKS POST CARCINOGENESIS

Type of Scores	8 weeks Iron-sufficient (+Fe)	8 weeks Iron-deficient (-Fe)	14 weeks Iron-sufficient (+Fe)
Palate	r = -0.05	r = 0.3	r = -0.1
Tongue	r = 0.2	r = 0.5	r = -0.2

TABLE 70

WEIGHT, SERUM RETINOL AND HAEMOGLOBIN OF CONTROL RATS (MEAN ± 1SD)

No. of rats	Iron Status	Weight at sacrifice (gm)	Serum retinol (μmol/l)	Haemoglobin (g/dl)
5	+Fe	331 ± 35	1.4 ± 0.3	14.0
5	-Fe	343 ± 20	0.96 ± 0.3	6.6
6	Control	340	1.6 ± 0.4	-

iron-sufficient or iron-deficient groups, and no changes were found when compared with the non-carcinogen group of rats. However group 5 of iron-sufficient rats became heavier than group 6 iron-deficient rats, whereas this was not observed between group 3 iron-sufficient and group 4 iron-deficient, which were treated similarly except for longer periods of application of carcinogen in groups 5 and 6. All animals had undetectable liver vitamin A at sacrifice perhaps because the animals used it to increase growth rate.

Only 2 of the animals developed a tumour, squamous cell carcinomas arising in the treated epithelium. Both of these were in the palates of iron-deficient animals, one of which had received carcinogen application for 8 weeks and the other for 14 weeks, and all other animals were in a pre-malignancy state. Prime et al (1986) found that iron status did not affect either initiation or promotion. He explained the failure to demonstrate this effect possibly since termination of the experiment after 40 weeks gave insufficient follow-up time for differences between the groups to become apparent. It would be wrong on the basis of this experiment to suggest iron-deficiency was not influencing initiation or the stage of promotion prior to the development of recognisable tumours. It is also possible, however, that the influence of iron-deficiency is in the later stages of carcinogenesis where the depressed cell mediated immunity known to be present in iron deficiency (Joynson et al 1972) may play a role in allowing overt malignancy to become manifest.

The iron status did not affect overall mean vitamin A concentration either at the end of carcinogen or at sacrifice. However, there was a correlation between the decrease in serum retinol and the decrease in haemoglobin (Figs 71, 72 and 74) and the correlation was greater for iron deficiency animals, while Figure 73 showed increase in serum retinol against decrease in haemoglobin in iron sufficient groups. One explanation for this may be that the low levels of serum retinol occur due to the technique used to make the animals iron deficient which may have caused a deficiency of nutrients other than iron. However, care was taken to ensure that the diet was sufficient. It is also possible that iron is required to maintain serum retinol. Mejia and Arroyave (1982) found in experiments a very highly significant positive correlation ($p < 0.001$) between the change in serum retinol and the change in serum level of iron ($r = 0.6$). On the other hand, retinol may be used up, in some way when animals are iron deficient. This is likely to be an effect of carcinogen rather than iron status, because no correlation was found in iron deficiency animals without carcinogen between the change of serum retinol and haemoglobin.

It is very difficult to explain the relationship between cancer and vitamin A in general, due to the different factors which affect this relationship. Further studies are needed. Sporn and his team (1976 and 1977) showed that high amounts of retinoids administered during the last period of tumour development can drastically lower tumour incidence. Here the retinoids act more like pharmodynamic than nutritional substances. This may explain why low serum retinol and liver vitamin A were found

in our studies, although we have supplied the animals with vitamin A containing diet. Nettesheim and Williams, 1976; Siddik et al, 1980 showed that vitamin A deficiency stimulated lung carcinogenesis induced by polycyclic hydrocarbons, possibly by altering the activities of enzymes involved in the metabolism of the carcinogens. There have been few studies showing the effect of low body vitamin A status on the enzymes involved in the metabolism of chemical carcinogens in liver. This may be relevant to our study.

The main feature of the present study is that retinol appears to have been exhaustively used up by the animal in response to the carcinogen. This loss of retinol from the liver occurs before visible oral tumour occurs. It may therefore be concluded that retinol is rapidly used up during growth of the animal, either during normal circumstances (Section 3.2.5.1) or when exposed to a carcinogen. Carcinogen however, leads to further requirements for retinol. It would therefore be of great interest to determine the effect of large vitamin A supplements on the rate of tumour development.

4) DISCUSSION

4.1) GENERAL DISCUSSION

The assays described in this study for analysis of retinol and its metabolites have several advantages over other currently available and widely used methods. In particular, use of HPLC provides the specificity lacking in most other methods. Other workers have used different mobile phases and extraction procedures as well as different chromatographic techniques (Section 2.1.3). Many of these methods have been investigated during the course of this study, but we were unable to achieve satisfactory accuracy and specificity. As far as we know, all previous studies did not achieve a separation of retinol and other metabolites at the same time using an isocratic HPLC system. Separation has been achieved in gradient HPLC system by Amedee-Manesme *et al* (1984); Roberts *et al* (1978) but this leads to some artifactual peaks at solvent changes and also requires a more complex and expensive HPLC system.

Great care was taken during the present study to ensure that all retinol metabolites were separated. It can therefore be confidently concluded that normal human or rat serum, and also serum from humans and animals with certain forms of cancer do not have any detectable retinoic acid or retinal in the serum. This will facilitate future studies on the effects of retinol analogues in prevention or treatment of certain cancers.

Although this assay could separate retinal sufficiently to permit the conclusion that it was not present in biological samples, it was not possible to obtain good recoveries. Further studies would be necessary if accurate quantitation of retinal is needed in other investigations.

This specificity has also led to greater accuracy in analysis of retinol. Most other methods are subject to interference, and the commonly used fluorescence method has been found to produce results which correlate poorly with specific results achieved by HPLC. This is because the fluorimetric method is less accurate since it does not differentiate between retinol, its metabolites and other interfering substances or drugs (Tables 4 and 34).

Despite this poorer specificity of the fluorimetric method, in many cases, the HPLC results were higher than the fluorimetric ones. This slightly surprising result is probably due to inefficient extraction of retinol during the fluorometric method and also to quenching of the fluorescent signal (Thompson et al 1971).

All of the analyses described can be performed on one column, using a simple isocratic system, only a slight change in solvent being required for analysis of all retinol metabolites. It should therefore be possible for any laboratory with access to HPLC to perform these analyses.

In plasma, retinol is found in two major forms, as a specific complex with RBP, and as an esterified component of chylomicra. The complex with RBP is formed in the liver, is associated with prealbumin in the plasma, and is involved in

transporting vitamin A in fasting plasma, whereas chylomicra are derived from the diet and are transported to the liver and to other organs (Ismadi and Olson, 1975). RBP binds only with free retinol and this explains the good correlation between RBP and retinol (by HPLC) consistently found during the present study. Of the vitamin A measured in fasting plasma less than 5% is normally in the form of retinyl esters (Smith and Goodman, 1976), and this was confirmed in the present study (Section 3.2.3).

Approximately 90% of vitamin A is stored in the liver of man and experimental animals. It is probable that the vitamin is distributed unevenly, with the right central lobe having the higher concentration and left lobe having relatively low concentration (Olson et al 1979).

This was confirmed in the present study in control rat livers, where the right lobe had approximately 100 ug/g; and the left lobe had 70 ug/g. For ease of handling, during the animal studies reported here, the overall liver content was measured, and this probably gives a better general assessment of liver stores. For the human studies, biopsy was taken from the most convenient area, which was usually the lower edge of the right lobe.

Clinical interest in evaluating vitamin A nutrition has increased in recent years, due to the importance of vitamin A in various disease states, especially severely ill patients. Measurements of liver retinol stores, mainly retinol palmitate, provide a good index of total body vitamin A (Section 1.8). Consistency in sampling site is necessary to reduce between-lobe variations. In this study, no correlation was found between

liver retinol metabolites and plasma retinol (Figures 47, 65 and 66). Therefore plasma measurements cannot be used to assess total body vitamin A content. It should however, be noted that liver free retinol (Figure 67) correlated with plasma retinol but this is of little value since liver retinol comprises only 3-23% of the total. There is a wide range in total liver retinol in biopsy samples from normal subjects (Table 52) which further complicates the interpretation of total body status.

Measurement of retinol in plasma is widely performed because of the ease of sampling and of analysis. As indicated above, this rarely reflects body vitamin A status, except in cases of severe vitamin A deficiency where both liver and plasma concentration may be affected by other factors which alter the plasma level of RBP. In protein malnutrition, RBP synthesis is reduced (Arroyave et al 1961; Friend et al 1961, Smith et al 1973 a,b) and therefore plasma retinol is low. When protein nutrition is improved, for example by provision of IVN (Section 3.2.4.2) the plasma retinol simultaneously increases. On the other hand, patients with an acute illness eg operation or infection, develop an acute-phase response with the preferential synthesis of certain liver proteins, such as CRP (Laurell 1985; Kushner et al 1981). In this situation RBP synthesis is reduced (Fleck et al 1985). There is therefore an inverse correlation between serum retinol (or RBP) concentration and serum CRP (Figures 35, 38-40). This may also be in part the explanation for the early fall in serum retinol in our rats treated with oral carcinogen, although acute-phase protein data is not available for these studies.

Liver stores of vitamin are usually sufficient for at least half a year in man (OlSon, 1969). Prolonged inadequate intake is therefore necessary to exhaust liver stores. In the rat studies, 2 and 5 weeks were required for liver and plasma concentrations respectively to fall to very low levels. Similar results were obtained by Dowling and Wald (1958).

In rats this depletion is of course accelerated because the animals were very young and rapidly growing. In grown man, it would take much longer for deficiency to develop. After serious illness, among the many nutritional problems, there may be a combination of protein depletion and retinol depletion. Treatment by oral or parenteral route is necessary to correct this situation.

There has been concern about the delivery of vitamin A to patients who require TPN. In particular various workers have shown that retinol may be degraded by exposure to light or losses may occur on the plastic of the infusion set (McKenna and Bieri, 1980; Gutcher et al 1984). In this study therefore we have confirmed using the specific HPLC method, that the retinol palmitate added to the TPN emulsion can be protected by maintenance of the emulsion at 4°C in the dark. Approximately 40% was lost in 24 hours or 80% lost in a week if the bottle was exposed to daylight. Care must therefore be taken during the infusion procedure. Recently it has been shown that more adsorbed losses to the infusion set occur with retinol supplements rather than retinol palmitate (Riggle and Brandt, 1986). We found minimal

loss of retinol palmitate in the present study. We also can therefore be confident that the daily dose of vitamin A was delivered to patients being intravenously fed.

There was a wide variety of intakes of vitamin A in patients receiving home IVN, ranging from 107 to 3214 µg/day retinol palmitate. This compares with the oral recommendation of 1,000 µg of retinol by the Food and Nutrition Board of the US Academy of Sciences (1980), and also 1,000 µg for intravenously fed patients, recommended by the Expert Committee of the American Medical Association (1979). It is apparent that there was no correlation between plasma retinol and the daily intake (Figure 30). However, none of the patients with an intravenous intake of > 2000 µg/day had low serum retinol concentration, confirming the adequacy of this amount. It is possible that if there is an excessive intake over prolonged periods, toxicity may occur. This was not observed in any of the current patients, although a case has been reported of vitamin A toxicity in a four and a half year old, receiving 3000 µg/day on home IVN for six weeks (Seibert et al 1981). Nonetheless, high serum retinol levels were observed in some of our patients (as high as 8.5 µmol/l) and similar high serum levels have been reported by Shils et al (1985) in some patients receiving 1000 µg retinol/day whilst on long-term home IVN.

It is important therefore to define the minimum vitamin A intake necessary to maintain optimal vitamin A status. Lowry et al (1978) showed that most postoperative patients maintained blood retinol concentration with an intake of 450-600 µg/day. On the other hand, in this study it was found that an IV intake of

107-750 µg/day, or oral supplements, did not consistently maintain vitamin A and RBP concentration in plasma. As previously discussed, this may not solely be due to inadequate retinol intake, but may also reflect an acute phase protein response. However, only one patient receiving home IVN had a CRP concentration greater than 30 mg/l, hence the extent of the acute phase response in this patient group was small. Indeed, Nichoalds et al (1977) found that 1000 µg retinol/day in post operative patients would not maintain plasma retinol concentration, but 1300 µg/day was more effective. Stromberg et al (1981) also found that 750 µg was sufficient to maintain serum vitamin A only in those patients who commenced IVN with normal biochemical measurements. Patients commencing with low vitamin A were not consistently improved at this amount of intake. Liver biopsy data would be of great value in assessing the overall adequacy of these different levels of intake, but this cannot be justified on ethical grounds.

In short-term TPN patients who received a mean retinol intake of 800 ug, the fall in plasma concentration may be due to net protein catabolism in response to infection and surgery rather than temporary interruption of supply of vitamin A. The increase of serum retinol concentration with TPN supplements was possibly due to an increase in RBP synthesis. The rise in serum retinol (and RBP) invariably occurred when patients were in positive N balance. The correlation between RBP and serum retinol was better than with other proteins eg albumin, transferrin. Arroyave and Calcano, (1979) found that infection lowers the serum level of retinol and RBP. They suggested that the

mechanisms by which serum retinol is reduced are related to an impaired release of retinol from the liver or to an increased turnover of RBP. This is consistent with the inverse correlation found in the study between CRP and RBP (and hence retinol).

Similar results were obtained for surgical patients receiving nutritional support enterally by fine bore nasogastric tube (Table 37). The average intake of these patients was 3391 µg/day (range 2860-4153). One patient received separately oral/enteral and IVN, and mean overall intake was 2668 µg/day. Vitamin A orally fed to adults is based upon the amount of retinol necessary to maintain an adequate blood concentration and to prevent all deficiency symptoms, plus an amount above the requirement to permit liver storage. Arroyave (1969), Popper and Steigmann (1943) explain that the signs of vitamin A deficiency may be detected due to immobilization of liver vitamin A in certain diseases as well as to malnutrition. No patients in the present study developed clinical signs of vitamin A deficiency.

The overall bioavailability of parenterally-administered pharmacological doses of vitamin A is unknown. Considering the probable loss of some vitamin A from TPN solutions and the degree of illness of the patients, it is surprising that there are not more reports of hypovitaminosis A in TPN. Perhaps the amount delivered to patients on short-term TPN is sufficient to prevent the development of deficiency symptoms over the time period studied, while patients on long-term TPN may not present with overt vitamin A deficiency (Sitren, 1984). Physical examination eg dark adaptation may be required over a prolonged period to detect sub-optimal intake. Most studies have assessed vitamin A

status in parenterally fed patients, only by measuring plasma retinol or RBP concentrations. These are less reliable indicators of vitamin A nutrition if the patients are well nourished with respect to energy and/or protein. The dependence of plasma vitamin A concentration on protein nutritional status and on inflammatory status make it difficult to assess adequacy of provision of vitamin A. Taken overall, the intakes of 800-1000 ug in short-term and in long-term studies seem adequate to prevent deficiency or depletion occurring. This would agree with the current recommendation of the FNB and of the AMA for seriously ill patients. Further studies including liver biopsy and dark adaptation testing are required to characterise the optimal intake in relation to disease and long term support.

It has been suggested according to various evidence that nutrition in general or specific nutritional components, in various parts of the world may play an important role in the causation and development of a number of types of cancer (Weisburger, 1985).

A major physiological role of vitamin A is to control cell differentiation. Since loss of cell differentiation is a basic feature of cancer, there is ample reason to suspect that intake of vitamin A may be related to cancer incidence (Willett and MacMahon, 1984).

For these reasons the association between serum and liver retinol levels with various cancer states, in animal and man have been studied in the present work. A major question has

been whether a low plasma vitamin A predisposes to the development of cancer, or whether it occurs in response to the presence of a cancer.

In most previous studies low circulating levels of vitamin A were observed in subjects who subsequently developed cancer of epithelial cell origin (Wald et al 1980, Kark et al 1981). However, Willett et al (1984) found mean serum retinol was similar for cases and controls (95% confidence limits for case-control difference). In eighteen subjects with subsequent lung cancer in their study, mean base-line retinol level was found to be higher than that in their matched controls. The explanation of these higher values is unknown.

Studies we performed on the effect of oral carcinogens in rats led to two different effects:- in one series of studies rats were treated with carcinogen for 8 or 14 weeks and then followed until 40 weeks, but in only 2 cases did an epithelial cancer develop. In most of the animals, atypia was present to a variable extent, and this was quantified (by Dr S Prime) using the system of Smith and Pindborg (1969). On the other hand, rats treated with carcinogen for prolonged periods almost invariably developed an oral carcinoma after 26-32 weeks of application. We are thus able to compare vitamin A status in animals in the pre-cancer situation with those which developed oral cancer.

Plasma retinol fell in response to carcinogen application, with those animals treated for 14 weeks having a lower plasma retinol than those treated with carcinogen for 8 weeks. Both groups had very low plasma retinol at the time of sacrifice (40 weeks) although tumours were not present, and

plasma concentrations of retinol were similar to those found in animals with oral cancers. Similarly liver vitamin A was not detected at the time of sacrifice in any of the animals treated with carcinogen whether or not an oral cancer had developed. This suggests that the vitamin A had been used by the rat in response to the carcinogen application, before a tumour had developed. The mechanism of this utilisation is not known. It can be speculated that vitamin A may be taken up from plasma by tissue exposed to a carcinogen in an attempt to maintain normal cell structure and function. This in turn may lead to increased mobilization of vitamin A from the liver.

This situation may relate to the increased incidence of cancer in individuals with vitamin A deficiency. Vitamin A deficiency in animals has been shown to increase susceptibility to chemical carcinogenesis in the respiratory tract (Nettesheim and Williams 1976); skin (Davies, 1967); bladder (Cohen et al 1976) and colon (Newberne and Rogers, 1973).

Wormsley, (1985) suggested that in pancreatic cancer after injury if at any time the replicating tissues are exposed to an initiating carcinogen, cancer may occur. Dogra et al (1985) proposed that vitamin A deficiency may enhance the process of tumourigenesis both at the initiation (the irreversible genetic alteration of a target cell by a carcinogen) and promotion phase (resulting in a phenotypic expression of that genetic alteration).

On the other hand studies in man, of patients with leukoplakia which is regarded as a pre-malignant disease (Silber, 1985) did not show a significant difference in serum vitamin A

compared with control (Table 50). It is noteworthy that patients with Crohn's Disease showed much lower serum retinol levels when compared with control or patients with leukoplakia. These patients are at risk of protein malnutrition as a result of the disease or resected small bowel. It would be of interest in leukoplakia patients to monitor the changes in serum retinol, over a period of careful observation of the leukoplakia especially if oral cancer developed.

Once a tumour has developed, low vitamin A status is probable. All rats with tumour in this study had low serum and liver vitamin A. Basu et al 1976; Atukorala et al 1979 reported that low serum retinol was associated with bronchial carcinoma. Ibrahim et al (1977) reported this association with carcinoma of oral cavity and oropharynx as well.

In the present study patients undergoing abdominal surgery for neoplastic disease of stomach or colon, did not have significantly different serum retinol from control. However, patients in whom the neoplasm was present in the rectum showed a significantly lower plasma retinol ($p < 0.05$) from control cases and those with gastric or other colon cancer. This difference is surprising but indicates that different tumours may be associated in different ways with vitamin A.

Although there was a tendency for patients with hepatic metastases at the time of operation to have lower plasma and liver retinol concentrations than those without metastases, there was no statistically significant difference. The liver stores of vitamin A are so varied that no difference was observed between patients with cancer and those with benign disease (Table 52).

Patients who had been treated surgically for colorectal cancer 7 days - 12 years before sampling, showed a significantly lower plasma retinol ($p < 0.01$) than controls and the difference was greater in males than in females (Table 39). This reduction was more apparent in patients with metastases (Figure 54) who were sampled within one month of operation. There were inadequate controls of patients without metastases sampled at the same time, to allow an assessment of the effect of tumour level. It was found that the significant difference described above disappears if samples taken within one month after operation are excluded. It is probably that this was due to the acute phase response with decreased RBP synthesis in the post operative period.

It has been suggested that there is a relationship between low cholesterol concentration and increased cancer risk. Williams et al (1981) suggested that it reflected the physiological response to early undiagnosed stages of cancer. Adams et al (1985) explained that it is possible that the association observed between low cholesterol concentration and increased risk is mediated through an unknown factor. In the present study, a correlation of $r = 0.5$ was found between cholesterol and serum retinol in the post-colorectal cancer situation and this was mainly observed in patients with liver metastases (Figures 51, 54 and 55). If the samples taken within one month after operation are excluded, there is no such correlation. This correlation in our study may therefore be due to the acute-phase response on apolipoproteins as well as on RBP.

Kark et al (1982) offered three conflicting hypotheses to explain the inverse association found in other studies. First, low cholesterol values before detection of cancer may be a result of the cancer process. Second, low cholesterol values may precede the development of cancer, but the association with the cancer is secondary. Third, low cholesterol values may precede the development of cancer and may be causually associated with occurrence of some forms of cancer. Until the major routes are understood, along with the kinetics of transfer, it may be impossible to make any correlation between cholesterol, beta carotene and risk of cancer (Peto, 1981). Adams et al (1985) in recent studies demonstrated a significant relationship between carotene and lipoprotein cholesterol in a healthy group of college students. It is probable therefore that the association of cancer with cholesterol is secondary, due to the correlation of retinol with cholesterol concentration.

On the other hand, Jagadeesan and Reddy (1978) found there was a significant correlation between vitamin A and haemoglobin concentration. Stored iron may be mobilized by improving the vitamin A nutritional status (Mejia and Arroyave, 1982). Overall we did not find any correlation or effect of iron status on vitamin A, since plasma retinol concentrations were similar in both iron-sufficient and iron-deficient groups. However, the observation that in rats treated with carcinogen for 8 weeks there was a significant inverse correlation between the magnitude of the fall in haemoglobin with the fall in plasma retinol (Figures 71 and 72) suggests that in some way, retinol may be involved in the maintenance of haemoglobin levels in

iron-deficient rats. This observation was not confirmed in rats treated with carcinogens for 14 weeks, suggesting that the effect of the carcinogen on retinol concentration is greater than the role of retinol in haemoglobin metabolism.

Although iron deficiency was associated with earlier development of oral tumours (mean 183 days) than iron sufficient rats (mean 229 days) when carcinogen was continuously applied, the shorter periods of carcinogen application did not demonstrate any significant difference in atypia scores for iron-sufficient and deficient rats. It is noteworthy that in all studies no difference in serum or liver retinol was observed between iron-sufficient or deficient rats. Thus any effects of iron-deficiency are unlikely to be mediated through vitamin A.

The role of vitamin A deficiency in tumourigenesis has been studied by several investigators. However, the exact mechanism by which vitamin A influences the process has not been delineated:-

i) Additional epidemiological and experimental studies are needed before definite conclusions can be drawn about the role of various forms of vitamin A in the aetiology of cancer. In the present study there is limited data showing the relationship between retinol status and cancer occurrence or subsequent cancer recurrence in patients who underwent surgery.

ii) The present study has indicated weak association between retinol and cholesterol, but prospective studies are needed to assess the relationships between carotene, cholesterol and cancer-risk to clarify the mechanisms involved.

iii) Vitamin A is altered by carcinogens during tumour induction (Nutrition Reviews, 1979). Further evidence for this has been obtained in the present study.

iv) Chytil and Ong and their co-workers (1978a,b) found specific intracellular retinoid-binding proteins, similar to steroid-binding proteins, in many tissues affected by vitamin A. They suggested that retinoids may act like steroids, which exert their effect on gene-expression, and this has considerable support (Sitren, 1984). Zile and Cullum (1983) suggested that the role of vitamin A in reproduction is mediated by specific intracellular protein carriers. If intracellular retinoid-binding protein mediates the effect of vitamin A, then its presence in various non-epithelial cells suggests that these cell types may also be targets. Further work on these binding proteins is required. In the case of this study the fall in serum retinol in rats may be due to carcinogens causing some effect on the number of receptors or in the amount of intracellular binding proteins.

v) Another possible mechanism of action of retinoids is their effect on glycoprotein synthesis. Deluca (1978) suggested that phosphorylated retinol or retinoic acid derivatives may participate in glycosylation of membrane glycoproteins by acting as carriers of monosaccharides across the hydrophobic lipid bilayer.

Several other mechanisms of action have been postulated. However, most of them have not been supported by subsequent investigations.

4.2) Conclusions

The main conclusions which can therefore be drawn from the present studies are as follows:-

i) A method has been developed to separate retinol and its derivatives qualitatively and quantitatively in serum, liver and IVN solutions.

ii) In normal man or rat and in those with certain types of tumour, the only serum retinoid detectable is retinol.

iii) There is a poor correlation between the commonly-used fluorometric assay and the specific HPLC assay. The fluorometric assay is only useful in the absence of interfering substances in the diet, such as pigment, and when all dietary triglyceride has been cleared from the circulation.

iv) The vitamin A in IVN solutions must be protected from degradation prior to infusion.

v) Plasma retinol can be maintained in patients receiving IVN or enteral nutrition. However the acute-phase reaction may interfere with the use of this measurement in assessing vitamin A status.

vi) Vitamin A deficiency in rats can be achieved with depletion of liver vitamin A stores within 2 weeks, followed by a fall in plasma retinol after about 5 weeks.

vii) Plasma retinol in rats falls in response to application of an oral carcinogen and low levels are obtained before a tumour develops.

viii) In man, leukoplakia was not associated with low serum retinol. In patients with intestinal cancer, low serum retinol was found in patients with rectal carcinoma and in those with

metastases. In liver there was no significant difference between patients with benign and malignant disease (with or without metastases) for retinol or its metabolites.

ix) Vitamin A status was not found to be associated with iron status in rats. The development of tumours was more rapid in iron-deficient rats and this is therefore unlikely to be mediated through the effects of retinol.

x) Serum cholesterol was associated with serum retinol in patients with metastatic cancer of rectum but this was mainly due to the acute phase response to surgery.

4.3) Further studies which might be performed to extend the above work might be:-

a) Liver biopsy samples would be valuable to assess the vitamin A stores in patients on various forms of nutritional support. This should be related to dark-adaptation studies.

b) The kinetics of change in serum and liver vitamin A in rats in response to carcinogen should be studied in more detail to determine the time course of depletion.

c) Longer term studies on the effects of carcinogen are needed.

d) The reason for the rapid fall in vitamin A after oral carcinogen should be studied eg by metabolism of labelled retinol.

e) Iron status in liver could be assessed in relation to vitamin A status.

f) The effects of vitamin A supplements on the response to carcinogen should be studied.

g) Studies on intracellular binding proteins in response to carcinogen would be of interest.

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