VITAMIN A AND LUNG CANCER

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by

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England.
The work presented in this thesis deals with a study of vitamin A and related factors in two groups of cancer patients with additional studies on experimental animals. Twenty-six patients with newly diagnosed, histologically proven lung cancer and nineteen patients with advanced testicular teratoma, many of whom had metastases in the lungs were selected for study.

The patients with lung cancer had significantly lower concentration of vitamin A in the serum than in the controls, and these low levels were related to low levels of retinol-binding protein (RBP), the carrier protein for vitamin A. In addition, subnormal serum zinc levels which were positively correlated with both vitamin A and RBP were also observed in these patients. These results are suggestive of a role for zinc in the vitamin A metabolism of these patients.

The effect of corticosteroids as a factor in affecting plasma vitamin A was investigated in healthy male Wistar-Albino rats. Corticosteroid treatment reduced the weight of the thymus and caused a loss of vitamin A. Concomitant administration of vitamin A did not significantly increase the size of the gland, but giving vitamin A increased its vitamin A content above that found in control animals. The thymus plays an important role in cell-mediated and other immune reactions. The significance of these observations with respect to depressed cell-mediated immunity in patients with cancer, and particularly lung cancer, has been discussed.
Studies on the vitamin A status of patients with metastatic testicular teratoma suggested that decreased synthesis of its carrier proteins, RBP and prealbumin were responsible for the lower vitamin A levels when compared to age-matched healthy male subjects. During each course of treatment with vinblastine and bleomycin, or cis-diamine dichloro platinum (II) together with the above drugs, the blood levels of vitamin A and the status with respect to water-soluble vitamins, thiamine and pyridoxine showed a marked fall. However, the overall effect from the beginning of the first course to the beginning of the fourth course was that blood levels of vitamin A were significantly higher at the end of treatment, whereas the status with respect to water-soluble vitamins remained the same or deteriorated further. The difference between the effects on vitamin A and water-soluble vitamins, thiamine and pyridoxine can be explained by the fact that blood levels of vitamin A are affected by liver function and this may have improved, during treatment, whereas the status with respect to thiamine and pyridoxine is dependent on dietary intake.

The adverse nutritional side effects of chemotherapy were also observed in healthy male Wistar-Albino rats. Treatment with vitamin A prior to administration of vinblastine reduced some of these side effects. The potential role of vitamin A as an adjuvant to chemotherapy has also been discussed.
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GENERAL INTRODUCTION
1.1 NUTRITION IN RELATION TO CANCER

Nutrition has long been thought to affect the pathogenesis of several cancers in man. Epidemiological evidence suggests that 50 - 90% of all cancers are of environmental origin (Ackermann, 1972; Rubin, 1973), and are therefore potentially preventable (Alcantara and Speckmann, 1976). Environmental pollution, radiation, excessive use of tobacco and food additives contribute to a small extent to the development of neoplasms. It is evident however, that a large percentage of certain cancers are related to nutritional factors. A current estimate points to the fact that 50% of all cancers in women and a third of all cancers in men are causatively related to nutritional factors (Wynder, 1976).

The relationship between diet, nutrition and cancer is very complex. Nutritional deficiencies, excesses or imbalances, may affect the development and progression of the cancer.

As early as 1914, Rous observed that the development of mammary tumours and metastases was delayed by food restriction. In a later study, Tannenbaum and Silverstone (1953) showed that many diverse types of neoplasms, spontaneous and chemically induced, respond to caloric deprivation by a reduction in tumour incidence and a delay in the appearance of the tumour. Lea (1966) showed a relationship between excessive fat consumption and deaths from certain types of cancer in man. Excessive intake of fat is associated with increased incidence of cancer of the breast and endometrium (Wynder et al., 1966). In experimental animals, carcinogenesis, particularly in the liver, could be modified through a change in the proportion or composition
of the dietary protein (Tannenbaum and Silverstone, 1953). It has been suggested that the fibre content of the diet may exert a protective effect against the development of cancer of the large bowel (Burkitt, 1971).

Deficiencies or excesses of several inorganic substances are also implicated in the genesis of tumours. For example, iodine deficiency may account for a relatively high rate of cancer of the thyroid in areas where goitre is endemic (Cowdry, 1968). A long standing deficiency of iron, as in the Plummer-Vinson syndrome is associated with cancer of the upper alimentary tract in man (Laarson et al., 1975). In experimental animals, dietary zinc deficiency has been shown to inhibit the development of Walker-256 carcinosarcoma and Lewis lung carcinoma, despite only small differences with weight-matched controls (De Wys and Pories, 1972).

A high correlation has been observed between alcohol consumption and cancer of the oral cavity, particularly the mouth, larynx and oesophagus (Wynder et al., 1957). Clinical observations show chronic alcoholism to be often associated with deficiencies of B vitamins (Vitale, 1971). The derivatives of riboflavin, a B vitamin, play an integral role in the respiratory system. Significant hyperplasia has been observed in the skin of riboflavin-deficient mice. Moreover, riboflavin deficiency enhances the susceptibility of mouse skin to tumour yield when treated with carcinogen and promoter as compared to control treatment (Wynder and Chan, 1970).

The association between vitamin A and cancer of epithelial tissues is perhaps the most exciting. About 80% of the cancers that
Vitamin A plays an important role in controlling the differentiation of epithelial tissues (Moore, 1967). Early studies in experimental animals revealed that vitamin A deficiency leads to metaplastic changes in epithelia, particularly in the respiratory, gastrointestinal and urogenital tracts (Wolbach and Howe, 1925). These metaplastic changes may be considered the first step in the transformation from a normal to a neoplastic tissue.

Furthermore, there is increasing epidemiological evidence pointing to an association between vitamin A and cancer of epithelial tissues, particularly the lung, in man. (Bjelke, 1975; Mettlin et al., 1979) Therefore, it is not surprising that a great deal of interest is focussed on the relationship of vitamin A to cancer of epithelial tissues.

1.2. VITAMIN A

1.2.1. Structure and metabolism of Vitamin A.

Vitamin A is a fat-soluble vitamin which exerts a number of important biological effects. It is necessary for vision (Wald, 1968), growth, reproduction (Thompson et al., 1964) and proper differentiation of epithelial tissues (De Luca and Wolf, 1968; Wong and Buck, 1971; Olson, 1972). The biologically active form in mammalian tissues is all trans-retinol (fig. 1.1). Its oxidized form retinal is involved in the visual cycle (Wald, 1968). Retinal can be further oxidised irreversibly to retinoic acid which is capable of promoting growth and differentiation of epithelial tissues, but is not active in vision (Dowling and Wald, 1960) and reproduction (Thompson et al., 1964).

The sources of vitamin A in the diet include retinyl esters from animal sources or its precursor β-carotene from plants. It
Fig. 1.1 Structures of vitamin A and its derivatives

\[ \text{β-carotene} \xrightarrow{\text{dioxigenase}} \text{all-trans retinoic acid (Vitamin A acid)} \]

\[ \text{all-trans retinal (Vitamin A aldehyde)} \]

\[ \text{visual purple of the eye (rhodopsin)} \]

\[ \text{II-Cis retinal} \]

\[ \text{isomerase} \]

\[ \text{dehydrogenase} \]

\[ \text{esterase} \]

\[ \text{Retinyl ester} \]

\[ R = \text{palmitate or acetate} \]
is absorbed from the upper intestine in the micellar form, and is transported to the liver mainly via the lymphatic pathway in association with chylomicrons (Huang and Goodman, 1965; Goodman et al., 1966). The chylomicron vitamin A is removed from the circulation by the liver (Goodman et al., 1965) where it is re-esterified and stored mainly as the palmitate. Unlike other vitamins, a fairly large amount of vitamin A is stored in the body, mainly in the liver.

Retinol may also be conjugated with UDP glucuronic acid in the liver to form glucuronides or may be oxidised to retinal and finally to retinoic acid. Retinoic acid also forms a glucuronide in the liver and these glucuronides, together with perhaps a small amount of free retinoic acid are excreted efficiently into the bile (Zachmann et al., 1966). Glucuronides of the bile are partially reabsorbed into the gut and transported again to the liver, thereby giving an enterohepatic circulation. Most of the biliary glucuronides however, seem to be hydrolyzed in the gut by enteric bacteria and excreted in the faeces as a mixture of free retinoic acid, possibly free retinol, the intact glucuronides and some other unidentified products (Olson, 1968). Metabolites of vitamin A are predominantly excreted via the faeces. Kidney also excretes an appreciable amount of vitamin A metabolites such as retinoyl glucuronides, but these compounds have not been well characterized (Nath and Olson, 1967; Sundaresan and Sundaresan, 1975).

The oxidised form of vitamin A, retinoic acid is not stored in the body (Dowling and Wald, 1960) and is transported in the plasma
bound to albumin (Smith et al., 1973). Vitamin A is mobilised from the liver as the free alcohol, retinol, bound to a specific protein — retinol-binding protein — RBP (Kanai et al., 1968). This is the form in which vitamin A is transported from the liver to peripheral tissues such as the eye, intestinal mucosa, gonads and salivary glands to supply their metabolic needs. It is likely that the delivery of vitamin A to tissues is controlled by processes which regulate the production and secretion of RBP by the liver (Goodman, 1974).

Retinol-binding protein is associated with the membrane components of the liver cell, mainly the Golgi fraction and the endoplasmic reticulum (Glover et al., 1974). Following its synthesis in the ribosomes, it is presumably transferred to the endoplasmic reticular membrane where it can pick up retinol released from esters by the action of liver esterase. It is then released into the circulation as the holoprotein (Glover et al., 1974). The apoprotein by itself is much less stable than the holoprotein (Glover et al., 1974).

Retinol-binding protein is a relatively small molecular weight protein, composed of a single polypeptide chain with a molecular weight of approximately 21,000 daltons (Raz et al., 1970; Peterson, 1971). It has a $\alpha_1$-mobility on electrophoresis and appears as three closely spaced bands on disc-gel electrophoresis, a non-fluorescent component corresponding to apo-RBP and two fluorescent bands corresponding to holo-RBP. The difference in mobilities arises from differences in the nett charge per unit density through the loss of amide groups or a terminal aminoacid during isolation (Glover, 1973). RBP contains a high proportion of aromatic amino acids and
dicarboxylic acids, some of which may exist as amides. It has a single binding site for a molecule of retinol (Kanai et al., 1968) and circulates mainly as the holoprotein (Glover, 1973).

Retinol-binding protein interacts strongly with prealbumin and circulates as a 1:1 molar complex of molecular weight of approximately 75,000 daltons in the plasma (Kanai et al., 1968; Raz et al., 1970 and Peterson, 1971). The interaction of retinol with RBP appears to be stabilised by the formation of RBP-prealbumin complex. Moreover, the formation of the complex may prevent the excretion of retinol-bound RBP by the kidney (Vahlquist, 1972). The formation of RBP-prealbumin complex is very sensitive to ionic strength with dissociation occurring at low ionic strength (Van Jaarsveld et al., 1973).

Pre-albumin is a tetrameric molecule, with a molecular weight of 54,000 daltons (Blake et al., 1971). Like RBP, prealbumin also contains a high proportion of aromatic amino acids, particularly tryptophan (Goodman, 1974). Although RBP normally circulates as a 1:1 molar complex with prealbumin, there is evidence to suggest that prealbumin may contain four binding sites for RBP (Van Jaarsveld et al., 1973). Thus it is possible that each subunit contains a single binding site for RBP. In addition, prealbumin is one of the three proteins involved in the transport of thyroid hormones in the plasma (Ingbar, 1963). Raz and Goodman (1969) and Van Jaarsveld et al. (1973) have shown that there is no interdependence of the binding of thyroxine and of RBP to prealbumin.

Peterson and co-workers using isolated intestinal mucosal cells
showed that the cellular uptake of retinol is mediated by a receptor (Rask and Peterson, 1976). The mucosal cells readily accumulate labelled retinol from its complex with RBP without concomitant cellular uptake of the protein itself. The membrane receptor seems to recognize the protein rather than the retinol. During the uptake of retinol, an altered form of RBP is generated, which cannot bind retinol and consequently prealbumin. It differs from holo-RBP, in that it lacks the terminal arginine residue (Rask et al., 1971). Heller and Chen (1977) using isolated pigment epithelial cells from bovine retina and labelled RBP also showed that the binding was at the cell surface without penetration of RBP into the cell.

Therefore, RBP is not only important for the transport of retinol in the blood, but is also an indispensable entity for recognition by the target cells and consequently for penetration of retinol through the plasma membrane.

A cellular binding protein which binds retinol was first discovered by Bashor et al., (1973). This protein has a stringent requirement for an alcohol group at C-15 and therefore does not bind retinal or retinoic acid. Later Ong and Chytil (1975) detected an intra-cellular binding protein specific for retinoic acid. These binding proteins are now called cellular retinol-binding protein and cellular retinoic acid-binding protein respectively. Cellular retinol-binding protein is a protein distinct from plasma RBP both in its antigenic as well as its chemical properties. Cellular retinol-binding protein is widely distributed in the adult rat (Bashor et al., 1973; Ong and Chytil, 1975 and Bashor and Chytil, 1975). It is not present in muscle and serum. It has been detected in man, rat, mouse
rabbit, cow, sheep and chick (Chytil and Ong, 1978). Retinoic acid binding protein is also present in some tissues. It is conceivable that in any tissue where vitamin A is known to control the differentiation of epithelia, such binding proteins might exist, fulfilling similar, if not identical functions.

1.2.2. **Deficiency, excess and the possible mode of action**

Very little is known about the mechanism of action of vitamin A except for its role in vision. Nevertheless, studies on vitamin A deficiency and excess have provided useful leads as to its function at the molecular level. Thompson and co-workers (1964) showed that vitamin A is necessary for reproduction. Injury to the reproductive organs, failure in reproduction and congenital malformations have been observed in vitamin A deficiency in experimental animals (Moore, 1967).

However, the most profound effect of vitamin A is its ability to control the differentiation of epithelial tissues. In rats fed a vitamin A deficient diet, normal epithelium is replaced by stratified keratinizing epithelium in various parts of the respiratory tract, alimentary tract, eyes and paraocular glands and the genito-urinary tract (Wolbach and Howe, 1925). Growth activity of the epithelium is not diminished, but greatly augmented, suggesting the acquisition of neoplastic properties. Addition of vitamin A caused a reversal of these changes (Wolbach and Howe, 1933). Epithelia of ectodermal, mesodermal and endodermal origin are similarly affected. The replacement epithelium, regardless of the original structure and function, is identical in all locations and is comparable in all its layers with the epidermis (Wolbach, 1954). An exception is the intestinal mucosa, where the number of mucus-secreting goblet cells is reduced considerably, but the epithelium does not keratinize (De Luca et al., 1969).
Vitamin A is also involved in the maintenance of mesenchymal structures (Fell and Mellanby, 1952; Fell and Dingle, 1963; Fell, 1970). Deficiency of vitamin A leads to defective bone modelling. Vitamin A deficiency also leads to degeneration of nerves and hydrocephalus (Moore, 1967). Also, numerous anatomical deformities may occur in the foetus as a result of lack of vitamin A in the maternal diet.

Ingestion of excessive amounts of vitamin A is also harmful and may lead to toxic side effects, such as headache, vomiting, diarrhoea. In hypervitaminosis A, mucous cell formation may occur in keratinizing epithelium. Excess of vitamin A also has profound effects on cartilage and bone tissue in vivo (Kochhar and Aydelotte, 1974) or in organ culture (Fell and Mellanby, 1952). Also, there is some evidence to suggest a teratological effect of excessive amounts of vitamin A in humans. Gal and co-workers (1972) observed elevated maternal serum vitamin A levels in some human central nervous system malformations.

It is now believed that some of the effects of hypervitaminosis A are due to labilisation of lysosomal membranes (Dingle and Lucy, 1965). Recent work suggests that the toxic manifestations of hypervitaminosis A occur when excessive amounts of vitamin A are presented to the cell membrane in association with lipoproteins, rather than specifically bound to RBP (Smith and Goodman, 1976). Thus RBP may not only regulate the supply of retinol to the tissues, but also protect tissues from the surface active properties of the vitamin. Not only retinol or retinyl esters, retinoic acid also has undesirable side effects because of its ability to damage lysosomal membranes (Goodman et al., 1974).

Although retinoic acid has the ability to control the differentiation of epithelial tissues, it cannot replace retinol in vision or reproduction. The failure of retinoic acid to replace retinol in all
its physiological functions seem to suggest that the effects of retinol and retinoic acid are not mediated by a single common intermediate, but that retinol and retinoic acid act as separate metabolic entities. This conclusion is further supported by the inability of cellular retinol binding protein to bind retinoic acid (Bashor et al., 1973).

Recently, De Luca and co-workers demonstrated the direct involvement of retinol and retinoic acid on the biosynthesis of glycoproteins, which are important constituents of the cell membrane. The mode of action of retinol and retinoic acid in the biosynthesis of glycoproteins has been reviewed elsewhere (De Luca, 1977). However, the possible significance of this observation is still not clear.

For a long time it has been speculated that the mechanism of action of vitamin A might be similar to that of steroid hormones (Bashor et al., 1973). They are believed to act on their target cells by binding first to specific proteins called receptors in the cytosol. Each protein has a high affinity and specificity for its steroid molecule. The ligand-protein complex is then translocated to the nucleus, where it interacts with chromatin, changing the expression of the genome. This interaction is manifested by alterations in nuclear RNA synthesis, resulting in changed differentiation. And indeed, altered nuclear RNA synthesis has been reported in vitamin A deficient and replete animals (Zachman, 1967; Zile and De Luca, 1970).

It is still not certain whether the cellular retinol and retinoic acid proteins are the receptors for vitamin A. However, Chytil and Ong (1978) have recently been successful in detecting specific interactions of the complex ($^3$H) retinol-cellular retinol-binding protein with nuclei isolated from livers of vitamin A deficient rats. No specific binding of free ($^3$H) retinol was observed indicating the necessity of cellular retinol-binding protein for the interaction to occur.
1.3. VITAMIN A AND CANCER IN EXPERIMENTAL ANIMALS

1.3.1. Vitamin A deficiency and chemical carcinogenesis

Vitamin A and its derivatives, collectively called retinoids, are important in controlling not only the normal differentiation of epithelial tissues, but also of premalignant epithelia (Moore, 1967). In fact, vitamin A deficiency, at least in the experimental animal, enhances the susceptibility of tissues to chemical carcinogenesis. Nettesheim and co-workers (1975) showed that low vitamin A intake increases the susceptibility of rats to develop lung tumours, on intra-tracheal instillation of 3-methyl cholangrene. A similar effect has also been observed in the induction of carcinoma of the bladder by N-(4-(5-nitro, 2-furyl) 2-thiazoly1) furamide-FANFT (Cohen et al., 1974). Also, greater incidence of colonic carcinoma in rats exposed to aflatoxin has been associated with low dietary and hepatic concentrations of vitamin A (Newberne and Rogers, 1973).

Harris and co-workers (1972) observed that both vitamin A deficiency and multiple intra-tracheal instillation of benzo(a)pyrene oxide caused squamous metaplasia in the tracheal epithelium of the hamster. By light microscopy, the squamous metaplasia without cell atypia caused by vitamin A deficiency is morphologically similar to that caused by carcinogen administration. However, significant differences were observed at the ultrastructural level.

1.3.2. Prevention of cancer by vitamin A derivatives (retinoids)

It is possible that vitamin A may prevent squamous metaplasia and the development of carcinomas which later arise from these precancerous changes. Chu and Malmgren (1965) observed that vitamin A
inhibits the induction of tumours of the forestomach and cervix of the Syrian hamster by carcinogenic polycyclic hydrocarbons. In a later study, Saffiotti et al. (1967), reported that treatment with high doses of retinyl palmitate markedly inhibited the induction of tracheo-bronchial squamous metaplasia and squamous cell tumours by intra-tracheal instillation of benzo(a)pyrene oxide in the Syrian hamster. Furthermore, vitamin A has also been shown to inhibit squamous metaplasia induced by benzo(a)pyrene in hamster trachea in organ culture (Crocker and Sanders, 1970). Bollag (1971) also observed a therapeutic effect of retinyl palmitate and of retinoic acid on skin tumours induced by dimethyl benzanthracene and croton oil. ...Shamberger (1971) in studies on mice using dimethyl benzanthracene-croton oil tumour promoting system confirmed the tumour inhibiting effect of vitamin A.

However, other workers did not observe a protective effect of vitamin A against the development of tumours (Levij and Polliack, 1968; Smith et al., 1975a). This may be due to the inability to achieve high blood levels of vitamin A on administration of large doses and to the toxic effect resulting from the deposition of large amounts of vitamin A in the liver. Retinoic acid which is not stored in the liver (Dowling and Wald, 1960) and is transported by albumin (Smith et al., 1973a) is not without its own undesirable side effects, particularly because of its ability to damage lysosomal membranes (Goodman et al., 1974). The need arises therefore, to synthesize a retinoid molecule which has the desired anticancer properties of retinoids, but lacks the toxic properties that had previously limited its usefulness. In fact, attempts are now being made to use synthetic
retinoids in the prevention of cancer (Sporn et al., 1976; Sporn, 1977; Bollag, 1977).

1.3.3. Synthetic retinoids and cancer prevention

The retinoid molecule could be modified either in the cyclohexenyl ring, the polar terminal group, or in the hydrocarbon side chain (fig 1.2A) without changing its anticancer action.

The first successful use of a synthetic retinoid was made by Bollag (1974). In the synthetic analogue used in this study, the cyclohexenyl ring was replaced by an aromatic trimethyl-methoxy phenyl (TMMP) ring and the terminal polar group of retinoic acid was replaced by an ethyl ester (fig 1.2B). The systemic administration of this compound resulted in a marked regression of carcinogen-induced skin papillomas and carcinomas in mice. It possesses a greater therapeutic ratio than retinoic acid, that is, a more favourable margin between the effective antitumour dose and the hypervitaminosis A producing dose. The TMMP derivative where the polar terminal group is replaced by an ethyl amide group is also active in the prevention of papillomas and carcinomas of mice and slightly less toxic (Bollag, 1975). Furthermore, the TMMP analogue of retinoic acid and its ethyl ester are also directly active in controlling the differentiation of tracheal epithelial cells in organ culture and cause reversal of keratinized squamous metaplastic lesions (Sporn et al., 1975). Another synthetic analogue, α-retinyl acetate, in which the double bond of the cyclohexene ring has been moved to the 4,5 position (fig 1.2C) also showed activity comparable to its natural β-analogue in controlling differentiation (Clamon et al., 1974).
In retinyl methyl ether, the polar terminal group is replaced by an ether group (fig 1.2.D). This compound was found to be as potent as retinol or retinyl acetate in supporting growth in the rat (Isler, 1949). Moreover, it is substantially less toxic than retinol or retinyl acetate on acute administration to the rat. In the rat, retinyl methyl ether shows a much greater ability than retinyl acetate to prevent mammary cancer induced by 7,12 dimethyl benz(a)anthracene (Sporn, 1977). It is possible that retinyl methyl ether may be cleaved to retinol in the body and be subsequently stored as esters in the liver. This factor may limit its chronic administration (Sporn, 1977).

Morton (1960) pointed out the importance of the side chain in the search for biologically active retinoids. Modification of the side chain is more difficult and very little progress has been made along these lines. In a long term study, Port and co-workers (1975) evaluated the ability of a synthetic analogue, 13-cis retinoic acid (fig 1.2.E) in preventing respiratory cancer in hamsters. In hamsters treated with a relatively low intra-tracheal dose of benzo(a)pyrene ferric oxide with no further treatment, there was a ten percent incidence of respiratory squamous carcinoma or in-situ carcinoma. The incidence of carcinomas was drastically curtailed by placing the animals on a life-time weekly dosage of 3 mg of 13-cis retinoic acid after completion of carcinogen dosing. Treatment with higher doses resulted in total prevention of the development of carcinomas. Furthermore, the treatment with even the high dose of 13-cis retinoic acid, did not result in manifest toxicity in the hamster, an equivalent dose of either all-trans retinoic acid or retinol would have
Figure 1.2. Structures of some retinoids

A Components of the retinoid molecule

\[
\begin{align*}
\text{hydrocarbon ring} & \\
\text{hydrocarbon side chain} & \\
\text{polar terminal group} & \\
\end{align*}
\]

\[R = -\text{CO OC}_2 \text{H}_5 - \text{ethyl ester} \quad -\text{CO NH C}_2 \text{H}_5 - \text{ethyl amide}\]

B The trimethyl methoxy phenyl (TMMP) analogue

C \(\alpha\)-Retinyl acetate

D Retinyl methyl ether

E 13-Cis retinoic acid
resulted in severe toxicity.

Sporn and co-workers (1976) have suggested the introduction of a fluorine or chlorine atom in appropriate positions of the side chain to obtain synthetic retinoids with increased biological activity and less toxicity.

1.3.4. Possible mode of action of retinoids

The mechanism by which retinoids exert a protective effect against insults by carcinogens is not known. Metabolic activation of these hydrocarbons is generally considered to be necessary for their carcinogenic activity (Miller, 1970). They are believed to be metabolised by microsomal mixed function oxidases through an epoxide intermediate (Hill and Shih, 1974). They suggested that vitamin A and other retinoids may be interfering with the activation of polycyclic hydrocarbons, by inhibiting microsomal mixed function oxidases. This inhibition has been demonstrated in the liver and lung tissues of mice and hamsters.

Chopra and Wilkoff (1976) in studies on cultured mouse prostate tissues, showed that retinoic acid inhibited and reversed the effect of both 3-methyl cholangrene (MCA) and methyl N-nitroso guanidine (MNNG). MCA is a carcinogen which requires activation, while MNNG does not require any metabolic activation (Marquardt et al., 1972). This implies that retinoic acid is acting at two different sites in altering the hyperplastic response produced by these two carcinogens (Chopra and Wilkoff, 1976).
The binding of carcinogen to DNA is believed to be necessary to induce anaplasia (Brookes and Lawley, 1964). Genta and his collaborators (1974), in studies on hamster trachea in organ culture, found that greater quantities of labelled benzo(a)pyrene was bound to DNA of vitamin A deficient hamsters. It is possible that vitamin A exerts its anticarcinogenic effect by preventing the binding of carcinogen to DNA.

Thus, it is clear that retinoids have the ability to prevent the development or progress of epithelial cancer in experimental animals. It seems pertinent therefore, to determine whether an analogous situation exists in man.

1.4. VITAMIN A AND CANCER IN MAN

Deficiency of plasma vitamin A has been observed in cancer of some epithelial tissues such as the stomach (Abels et al., 1941), cervix (Wynder, 1969), oro-pharynx (Ibrahim et al., 1978) and respiratory tract (Basu et al., 1976).

Dijkstra (1963) in an investigation of 330 consecutive patients with proven bronchial carcinoma, seen in the northern parts of Netherland suggested that those born in winter months have a greater chance of getting bronchial carcinoma than those born in the summer months. The foetus at birth has a low supply of vitamin A and in winter months the level of vitamin A in cow's milk is at its lowest. Dijkstra speculates that if the newborn infant is fed on cow's milk that is low in vitamin A, the vitamin A level of the infant may remain
low at the critical period of active bronchial development. This may result in squamous metaplasia which later predisposes the individual to develop bronchial carcinoma, when exposed to further bronchial insults.

In a survey of dietary habits and cigarette smoking in 8278 residents of Norway, Bjelke (1975) observed that dietary vitamin A intake is negatively associated with lung cancer at all levels of cigarette smoking. The negative association between dietary vitamin A intake and lung cancer is more clearly expressed in histologically proven bronchial carcinomas other than adenocarcinoma. The index of vitamin A used in this study included only a measure of the consumption of foods such as vegetables (especially carrots), milk and eggs. Failure to obtain information about the consumption of vitamin pills and liver, a rich source of vitamin A, casts some doubt on the findings.

In a very recent study (Mettlin et al., 1979), retrospective dietary and smoking data were gathered from 292 male patients with lung cancer and 801 control patients with non-respiratory, non-neoplastic diseases, and lung cancer patients had a lower computed index of vitamin A than controls. Furthermore, the relative risk of lung cancer was reduced with high vitamin A intake and the reduction was most evident in heavy smokers.

Thus it would seem to be of great importance to study the association between vitamin A and primary and metastatic lung cancer.
1.5. **CANCER**

A cancer is characterised by a group of cells that are behaving in an abnormal manner, escaping from some, if not all, the normal mechanisms which control cellular growth and anatomical arrangement. The transformation of a normal cell into a cancer cell may be the result of a number of complex interactions. The cells of malignant tumours, unlike those of benign tumours, invade local tissues and spread via the lymphatics, blood stream and body cavities to form secondary tumours or metastases remote from the site of origin. Most patients with malignant disease die from the harmful effects of disseminated disease rather than from the primary tumour itself.

Lung cancer and testicular teratomas, which often metastasise to the lung in the early stages, will be discussed in detail.

1.5.1. **Lung cancer**

Lung cancer was a rare disease fifty years ago. Today, it is the most common cause of death from cancer in man (fig 1.3) and now accounts for 6% of all deaths. Lung cancer is more common in men than in women, but, its incidence is rapidly increasing in women too (fig 1.4).

**Aetiology**

Doll and co-workers in a 20 year survey of about 30,000 British doctors concluded that smoking is an important factor in the production of carcinoma of the lung (Doll, 1950; Doll and Hill, 1964; Doll and Peto, 1976). The ratio of the death rate among cigarette smokers to that among life long non-smokers of comparable age for men under 70 years
Fig. 1.3. Trend in mortality from common cancers in men 1911-1971, standardised for age (from Doll, 1974).

Fig. 1.4. Trend in crude death rate from lung cancer 1911-1971, by sex, showing state at time of MRC Conference (from Doll, 1974).
was 2:1 and for men over 70 years 1.5:1. These ratios suggest that between a half and a third of all cigarette smokers will die if the excess death rates are actually caused by smoking. Wynder and co-workers (1970) in a retrospective epidemiological investigation of 350 lung cancer patients confirmed the close association between cigarette smoking and lung cancer, particularly of the squamous and oat cell types.

A similar association between lung cancer and cigarette smoking has also been observed in a retrospective study of 108 female lung cancer patients (Wynder, 1973). As women adopt smoking habits similar to those of men death rates from lung cancer continue to increase. However, smoking cigarettes with a low tar yield is expected to considerably decrease the incidence of lung cancer.

Among cigarette smokers, smoking more than one pack a day, a longer history of smoking, earlier starting age, inhalation and smoking non-filter cigarettes were significantly associated with greater morbidity ratios. Doll (1977) in a study of the observation of mortality rates suggests that, in the absence of smoking, the mortality from lung cancer in men might be reduced by 90 - 95%.

The mode of action of cigarette smoke is not clear. Tobacco or cigarette smoke contains a high proportion of polynuclear aromatic hydrocarbons, some of which have been shown to be potent carcinogens in experimental animals (Wynder et al., 1963). Two of the most important carcinogens found in cigarette smoke are benzo(a)pyrene (3.9 ± 0.3 µg in smoke from 100 cigarettes) and dibenzanthracene
(0.4 μg in smoke from 100 cigarettes). Cigarette smoke also contains co-carcinogens. Therefore the relation of lung cancer to cigarette smoke may represent the summation of a number of different substances, including carcinogens and co-carcinogens.

Another less important contributory factor to the development of lung neoplasms is atmospheric pollution. The combustion of coal releases carcinogenic agents such as benzo(a)pyrene and arsenic. Thus, it has been observed that lung cancer is more common in urban areas than in rural districts. Respiratory tract cancers are also common in workers in asbestos industry, presumably as a result of local irritation.

The polynuclear aromatic hydrocarbons present in cigarette smoke are broken down by the enzyme aryl hydrocarbon hydroxylase to carcinogenic epoxides (Emery et al., 1978; Korsgaard and Trell, 1978). Not all smokers get lung cancer. It is possible that certain individuals are constitutionally predisposed to the disease if they smoke. The inducibility of aryl hydrocarbon hydroxylase in peripheral blood lymphocytes of patients with squamous cell carcinoma of the lung was significantly higher when compared to controls matched for age, social class and smoking habits (Emery et al., 1978).

The importance of nutritional factors, particularly vitamin A, in the aetiology of lung cancer is now being realised, and has been discussed earlier. Also, deficiency of vitamin A may predispose smokers to develop lung cancer. In liver and lung tissues of mice and hamsters, vitamin A has been shown to inhibit the activity of
enzymes that metabolize polynuclear aromatic hydrocarbons (Hill and Shih, 1974). Thus, the risk of developing lung cancer may be reduced in the presence of adequate vitamin A. In fact, a recent study has revealed that high dietary intake of vitamin A reduces the relative risk of lung cancer, particularly in heavy smokers (Mettlin et al., 1979).

Pathology

Bronchial carcinoma is the commonest primary tumour of the lung. Usually, the tumour forms a mass surrounding the main bronchus to the lung or to one lobe (hilar type). Lymphatic spread often produces further nodules in the mucosa towards the bifurcation of the trachea. The carcinoma narrows the affected bronchus causing obstruction. Less frequently, the tumour may originate from a peripheral bronchus (peripheral type). Early and widespread invasion of the lymphatics occurs and may extend to the pleura.

Metastases are widespread and may involve virtually any organ in the body. There is a special tendency for the formation of secondary tumours in the brain, which may overshadow the primary bronchial tumour clinically. Metastases in bone are common, the thoracic vertebrae being frequently involved. Widespread metastases may occur from a small and clinically silent bronchial carcinoma.

Histological types

There are four main histological types of bronchial carcinoma, namely:

(1) squamous cell carcinoma
(2) oat cell carcinoma
(3) adeno carcinoma
(4) undifferentiated carcinoma

The structures may be mixed in some tumours.

Squamous cell carcinoma is the most common form of bronchial cancer, representing approximately 45% of all lung malignancies. It presents macroscopically as a dense, whitish, hilar mass, often with a flaky surface. It arises from the bronchial epithelium which has undergone squamous metaplasia, areas of which are seen in the bronchial mucosa of cigarette smokers (Auerbach et al., 1961) and patients with chronic bronchitis.

Oat cell carcinomas account for about 35% of the total number of cases of lung cancer. They usually arise near the hilum of the lung and are composed of very short, darkly staining, spindle cells that may appear oval or round. Some oat cell tumours secrete ACTH (adreno-corticotrophic hormone) which causes adreno-cortical hyperplasia (Odell, 1974).

Adenocarcinoma is the least common of the four main types and is composed of cuboidal or columnar cells, some of which secrete mucus. Sometimes these tumours may have a distinct papillary structure or they may be more schirrous. Adenocarcinomas account for 5 - 10% of primary lung cancers and more than half arise in the more peripheral intra-pulmonary sites.

Clinical features

The vast majority of patients with carcinoma of the lung present
with one or more symptoms associated with the lesion. Only 5% of the patients are asymptomatic and have a tumour suspected on routine physical examination or chest radiographs. The symptoms produced by centrally located lesions are frequently related to bronchial invasion, irritation, ulceration or partial occlusion. These include cough, haemoptysis and wheezing. The cough is persistent, progressive and usually non-productive. A peripheral lesion may become quite large with invasion of the pleura and chest wall before it is diagnosed, and associated pain may be the symptom in these patients.

The prognosis of lung cancer patients is very poor and about 80% of patients die within a year of diagnosis. About 30% of patients with small peripheral lesions in the lung survive more than five years after the operation, whereas the comparable figure for all lung cancer patients is 5 - 10%. Survival is longer in patients with squamous cell carcinoma and much shorter in those with undifferentiated and adenocarcinoma (Clinical Oncology, 1978).

1.5.2. Testicular teratoma: Metastatic lung cancer

Lung is a great filter of the blood stream and it is not surprising that a wide variety of tumours may give rise to pulmonary metastases. Testicular teratoma is a highly malignant tumour which often metastasises to the lung in the early stages. Recently it has been pointed out that about 20% of patients with clinical Stage I disease harbour occult pulmonary metastases. (Editorial, Cancer topics, 1979). Testicular tumours have now been recognised as one of the commoner malignant diseases in males up to the age of 35 years. (Table 1.1)
In this age group, more than 95% of the tumours are germinal cell tumours, namely seminomas and teratomas. Although seminomas are more common, teratomas are also of great importance as they are much more malignant than seminomas.

Table 1.1 Mortality from selected malignancies for males by age, England and Wales, 1970.

<table>
<thead>
<tr>
<th>Site</th>
<th>Death rate/Incidence per million population</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Age</td>
</tr>
<tr>
<td></td>
<td>15-24</td>
</tr>
<tr>
<td>Testis</td>
<td>11/28</td>
</tr>
<tr>
<td>Hodgkin's disease</td>
<td>16/38</td>
</tr>
<tr>
<td>Myeloid leukaemia</td>
<td>12/14</td>
</tr>
<tr>
<td>Trachea; bronchus; lung</td>
<td>3/3</td>
</tr>
</tbody>
</table>


A teratomatous tumour consists of a chaotic array of ectodermal, endodermal and mesodermal derivatives of varying degrees of differentiation and malignancy, along with areas of typical embryonic carcinoma. Teratoid elements frequently include smooth muscle, connective tissue, cartilage, bone, gastro-intestinal and respiratory epithelium, nervous and cutaneous structures. In a majority of patients, the secondaries that arise from the primary tumour are also teratomatous, with varying degrees of differentiation. Metastases usually occur in the following sites in the order of frequency: lung, abdominal lymph nodes, nodes above the diaphragm, liver, brain, kidney, bone and other sites (Pugh and Cameron, 1976).
Pathological staging of teratomas suggested by Pugh and Cameron (1976) is shown in table 1.2.

Table 1.2. Pathological staging of non-seminomatous germinal cell tumours of the testes.

<table>
<thead>
<tr>
<th>Teratoma</th>
<th>Malignant</th>
<th>Malignant</th>
<th>Malignant</th>
<th>Malignant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Differentiated</td>
<td>Teratoma</td>
<td>Teratoma</td>
<td>Teratoma</td>
<td>Trophoblastic</td>
</tr>
<tr>
<td>Intermediate</td>
<td>Undifferentiated</td>
<td></td>
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</tbody>
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Recently, it has been discovered that the tumour cells from a substantial number of patients synthesize marker proteins such as α-fetoprotein (αFP) and β-human chorionic gonadotrophin (βHCG) and a specific testicular isoenzyme of lactic dehydrogenase (LDH). Alphafetoprotein and β-HCG have been demonstrated within the tumour cell cytoplasm using the immuno-peroxidase technique (Heydermann et al., 1976). However, this technique has still not improved the pathological staging of tumours. After the histological type, the clinical stage (Table 1.3) and bulk of the tumour are the most important predictors of long term outcome (Tyrrell and Peckham, 1976).

Table 1.3. Staging of testicular tumours. (after Peckham and McElwain, 1976).

Stage

I  Tumour confined to the testis.

II Metastases to para-aortic region but no metastases above diaphragm.

III Metastases above diaphragm but only detectable in lymph nodes.

IV Metastases in non-lymphoid organs, lung, liver, bone, skin and central nervous system.
However, early detection does not necessarily mean better prognosis. In fact, Miller and Seljeldid (1971) have pointed out that some cases with a longer history may have a better prognosis. Nevertheless, early diagnosis may make the tumour more amenable to treatment.

**1.6 NUTRITIONAL PROBLEMS OF THE CANCER PATIENT**

The uncontrolled growth of the tumour occurring in active neoplastic disease may have unfavourable metabolic consequences. The growing tumour may derange the metabolism of the host because of its own requirement for nutrients (Basu et al., 1973). In fact, many patients with cancer are often malnourished, either as a result of the disease or as a consequence of increasingly aggressive forms of treatment often of a multiple nature (Dickerson and Tredger, 1977; Dickerson and Basu, 1978; Leading article, Brit. Med. J., 1979).

Sometimes the tumour may cause physical interference with strategic organs. Thus, patients with cancer of the upper alimentary tract are often malnourished as a result of decreased food intake due to partial or complete obstruction. The involvement of the pancreas, pancreatic duct or common bile duct may lead to impaired digestion or absorption of fats or fat-soluble vitamins.

In contrast, certain metabolic abnormalities occur which are not the immediate result of mechanical interference with recognisable structures (Costa, 1963). This poorly understood state of ill-health observed in some patients with advanced cancer is termed cachexia. It is characterised by loss of body weight, associated with anorexia,
increased basal metabolic rate and energy expenditure, marked weakness, loss of body fat and protein, anaemia, water and electrolyte abnormalities (Theologides, 1977). This condition is more common in patients with tumours of the alimentary tract than in patients with breast and lung cancer (Dickerson and Basu, 1978). Cachexia is a genuine clinical syndrome and may be reversed by surgical removal of the total malignant growth or when complete remission of the tumour is achieved by radio-or chemo-therapy (Theologides, 1977).

The cause of cachexia is at present unknown. It has been suggested that cancers frequently produce peptides or other small molecules which have been called toxohormones and that these act upon the tissues of the host to release amino acids into the metabolic pool, which are used for continued tumour growth (Hall, 1974). Theologides (1974) has suggested that these substances enter host cells, and through successive transitions, activations and inactivations of normal enzyme systems throw the metabolism of the host into a chaotic state. This metabolic chaos results in increased release of amino acids into the metabolic pool. Studies on experimental animals have shown that the tumour serves as a 'nitrogen-trap' incorporating amino acids into the tumour in an essentially one-way passage from the metabolic pool to the tumour (Mider, 1953). Hypoalbuminaemia has been observed in many cancer patients (Mider et al., 1950; Calmon, 1978) and this is considered to be due mainly to decreased albumin synthesis (Steinfeld, 1960; Mariani et al., 1976) and partly to protein losing gastroenteropathies (Waldmann et al., 1974).
Watkin (1959) showed extensive losses in body fat in the cancer patient. The mechanism by which the tumour induces lipid mobilisation is not clear. Cancer patients also show altered carbohydrate metabolism, connected to the derangement of protein and lipid metabolism. The pathway of gluconeogenesis is greatly augmented (Gold, 1974). Also, some patients with neoplastic disease have a decreased sensitivity to insulin (Marks and Bishop, 1957).

Deficiencies of vitamins other than vitamin A have also been observed in patients with cancer at different sites in the body (Dickerson and Basu, 1977). A significant proportion of patients with advanced malignant disease have been found to be at risk of thiamine deficiency, as judged by the stimulating effect of transketolase enzyme activity by thiamine pyrophosphate (Basu et al., 1974a). The requirement of folic acid is increased in patients with cancer (Einhorn and Reizenstein, 1966). This observation is supported by the demonstration of increased folic acid reductase activity, following infection of mouse kidney cell cultures with polyoma virus (Frearson et al., 1966). Also, Rao et al. (1965) have reported lower serum folic acid in patients bearing malignant tumours as compared to the control group. Abnormalities in vitamin B₆ and tryptophan metabolism have been observed in a significant number of patients with Hodgkin's disease and in some patients with carcinoma of the breast (Chabner et al., 1970).

Ascorbic acid plays a number of important roles in the body. Kramsner and Dymock (1974) have shown that tissue stores of ascorbic acid are depleted in patients with advanced malignant disease. Low
Leucocyte ascorbic acid levels have been found in patients with breast cancer and lowest values were found in patients with skeletal metastases (Basu et al., 1974b). The ascorbic acid concentration in the plasma and leucocytes of children with acute lymphoblastic leukaemia were significantly lower compared to normal age matched children (Kakar et al., 1975).

These nutritional deficiencies may be aggravated by the specific treatment used to control the disease, namely surgery, radiotherapy or chemotherapy.

1.7. TREATMENT OF CANCER AND ITS EFFECT ON NUTRITION

Surgery can cure the disease only when it is localised to the tissue of origin and its regional draining lymph nodes. Radiation therapy is often employed in the treatment of localised, but inoperable tumours. Systemic treatment with drugs or hormones may be used when the disease is in the disseminated, inoperable stages. More recently, immunotherapy has been employed either by itself, or in combination with other modes of therapy in the treatment of certain types of cancer. Multimodal therapy is currently favoured, as it yields better survival rates and more effective tumour remission.

1.7.1. Treatment of lung cancer.

Surgical extirpation of bronchogenic carcinoma remains the only consistent method of achieving a cure (Ashor et al., 1975). Lobectomy,
when technically feasible, is to be preferred, since it gives survival rates as good as pneumonectomy (Higgins and Beefe, 1967). Moreover, it is specially advisable when pulmonary function is borderline. The hilar lymph nodes should be removed with lobectomy or pneumonectomy; however, if they are involved the chances of cure are remote.

Oat cell carcinoma cannot be cured surgically (Medical Research Council, 1966). The disease invariably extends outside the chest and there is bone marrow invasion in 50% of the cases. Treatment consists of radiation to the lung and intensive chemotherapy for metastatic disease.

Of all patients with pulmonary malignancy, 50% or more of the tumours are found to be unresectable at the time of the patient's initial hospital evaluation (Thompson, 1967). Radiation therapy may be useful for palliation in inoperable patients with symptoms (Roswit et al., 1968), but is seldom indicated in the symptom-free patient with disseminated disease. (Durrant et al., 1971). In rare cases it may be curative in patients with localised disease who cannot tolerate surgery for medical reasons.

Radiation therapy often induces significant anorexia and loss, or alteration, of sensation of taste which may considerably decrease the food intake. Many patients suffer considerable weight loss between initiation and completion of therapy.

Chemotherapy is used primarily as an adjuvant following surgical resection or as a palliative tool in patients with unresectable or metastatic carcinoma. The results of single alkylating agents such
as cyclophosphamide and the nitrosoureas have been disappointing (Shields et al., 1974). Considerable enthusiasm has been generated for intermittent long-term adjuvant chemotherapy, particularly when given in combination with immuno- or radiotherapy. (Israel and Depierre, 1976; Donovan et al., 1976). Encouraging results have recently been reported using a regimen of combination chemotherapy with radiotherapy to the chest lesion and prophylactic brain irradiation in oat cell carcinoma (Greco et al., 1978).

Long term survival of occasional patients in whom tumour has been left behind after operation and the well-known fact that patients with tumours of similar extent, situation and histological type may progress at widely different rates suggests that the immune responsiveness of the individual plays an important part in determining the progress of the cancer. Furthermore, some indices of immune competence are depressed in lung cancer patients and the severity of this depression correlates inversely with survival rate (Liebler et al., 1977). There is, therefore, considerable interest at present on the role of immuno-stimulants in the treatment of lung cancer. Promising results have been claimed for both BCG and levamisole as adjuvants to surgery, but it is too early to assess whether these agents have a significant role.

1.7.2. Treatment of testicular teratoma.

Until the introduction of effective chemotherapy, radiotherapy or surgery provided the only hope of cure for the patient with malignant teratoma of the testis. Thus, more than 50% of the patients died of uncontrolled malignancy and cures, with rare exceptions, were confined
to patients with relatively early stage of the disease. The demonstration that a small proportion of patients with advanced disease could be cured with single agent chemotherapy, particularly with actinomycin D (MacKenzie, 1966), provided the first hint that teratomas, recognised as chemosensitive tumours for a long time might eventually prove to be curable by chemotherapy.

The primary testicular tumour is treated by orchidectomy, with high ligation of the spermatic cord. Prophylactic radiotherapy is often given to the normal para-aortic lymph nodes in stage I disease and to the mediastinal lymph nodes in stage II disease (Peckham and McElwain, 1976). Even with the best results from surgery or radiotherapy, 25% of stage I and II tumours will recur at some stage and will also need chemotherapy. Li and co-workers (1960) first reported some success against disseminated testicular cancer with a combination of actinomycin D, chlorambucil and methotrexate. However, there was no major change in survival rates until the introduction of a combination of vinblastine and bleomycin (Samuels et al., 1976). They reported a greatly improved overall response rate of 75% and a complete response of 32% in 50 patients with stage III testicular cancer. Spiegel et al. (1978) also showed that the combination of vinblastine and bleomycin gave a higher response rate than vincristine (a non-myelosuppressive drug), actinomycin D and bleomycin. Very recently, Peckham et al. (1979) have reported disease free survival rates in 81% of patients with bulky abdominal nodes and those with limited lung disease.
platinum II (cis DDP) would produce tumour regression in patients with metastatic testicular tumours. Nine of the 11 patients they treated responded, 3 of whom had complete disappearance of the tumour. Subsequent work by Einhorn and co-workers (1977) incorporating cis-DDP has shown that the combination of vinblastine, bleomycin and cis-DDP is an effective regimen and that complete remission confers a high probability of long-term disease-free survival and probably cure. These recent developments have greatly transformed the prospect for the patient with metastatic testicular teratoma.

However, the response to chemo- or radio therapy is largely influenced by tumour volume and the prognosis is decreased in bulky metastatic disease. Also, these drug combinations cause toxic side effects, which may adversely affect the health and nutritional status of the patient.

1.7.3. Drugs used in chemotherapy.

(a) Bleomycin

Bleomycin is an antitumour antibiotic first isolated by Umezawa and co-workers (1966) from cultures of *Streptomyces verticillus*. More than 200 different bleomycins have been isolated and characterized as complex, basic glycopeptides (Umezawa, 1971). The various bleomycins differ from each other only in the terminal cation moiety, which consists of an amine or polyamine moiety (Fig 1.5). Bleomycin A2 is used clinically in the treatment of oesophageal carcinoma, Hodgkin's disease, non-Hodgkin's Lymphomas, squamous cell carcinoma of head and neck, uterine cervix and testicular carcinoma (Carter et al., 1977).
Bleomycin first removes the thymine from native DNA in vitro, by hydrolysis of the N-glycosidic bond, without modifying the deoxyribose moiety (Fig 1.6). In a second step, single strand scissions occur at the sites of non-glycosidic deoxyribose moieties, resulting in the formation of 3'-hydroxy and 5'-phosphate termini (Muller and Zahn, 1977). It is suggested that bleomycin is bound to DNA by interaction of the positively charged amine moiety with a negatively charged phosphate group in DNA; intercalation seems to be involved in the binding.

In intact cell systems, bleomycin reduces DNA synthesis selectively by the induction of single strand breakages; RNA synthesis as well as protein synthesis is unaffected. Cell progression is inhibited by bleomycin at the end of the S-phase and early half of the G2 phase, thus showing the drug to be a possible synchronizing agent (Muller and Zahn, 1977). Bleomycin is detoxified by a bleomycin-inactivating enzyme which is present in all tissues other than the lungs and skin (Umezawa, 1970). The concentration of inactivating enzyme was found to be significantly lower in squamous cell carcinoma than in sarcoma of mice (Umezawa, 1973). Thus, it may be effective against squamous cell carcinoma. The activity of bleomycin is dependent upon uptake, inactivation and activation leading to tissue and organ specific actions.

(b) Vinblastine

Vinblastine sulphate (Velban), used in combination with bleomycin, is an alkaloid (Fig 1.7) originally isolated from the periwinkle, *Vinca rosea* (Johnson et al., 1960). The mechanism of action of vinblastine is not very clear. In low concentration, vinblastine
Figure 1.5 Structure of bleomycin

\[ \text{R = terminal amine or polyamine group} \]

Figure 1.6 Schematic representation of bleomycin caused degradation of DNA (from Muller and Zahn, 1977).
arrests cell division in the metaphase (Palmer et al., 1960). It causes reversible disruption of the mitotic spindle (Lettre, 1966).

The spindle fibres are long strands of microtubules connecting the two centrioles in the dividing cell to the kinetochores of the chromosomes. The spindle fibres direct the segregation of the sister chromatids to the opposite poles during anaphase. The spindle fibres contain microtubular proteins such as tubulin. Vinblastine and other Vinca alkaloids have been shown to interact with tubulin (Creasy, 1975). These binding interactions may cause dissolution of the spindle, thus preventing migration of the sister chromatids (Fig 1.8). However, there is no conclusive evidence to suggest that cytotoxicity is due entirely to the ability of Vinca alkaloids to cause metaphase arrest. Inhibition of nucleic acid synthesis by Vinca alkaloids has also been reported (Jones et al., 1966). Creasy (1978) suggests that inhibition of biosynthetic processes and damage to macromolecules on exposure to Vinca alkaloids may bring about cell death.

(c) Cis-diamine dichloro platinum (II)

The recent discovery of anti-tumour activity in square planar co-ordination complexes of platinum has revolutionized cancer chemotherapy (Rosenberg et al., 1969). Cis-diamine dichloro platinum (II) - Cis DDP (Fig. 1.9) is now in clinical use against a wide variety of cancers in man, including testicular malignancies (Rosenberg, 1971; Higby et al., 1974).
Figure 1.7 Structure of vinblastine

Figure 1.8 Diagrammatic representation of the mitotic spindle and the site of action of vinblastine (and vincristine) (from, Pratt, 1973).
Fig. 1.9. Structure of cis-diamine dichloro platinum (II) - cis-DDP.

Its mode of action is still not clear. The sites of the primary lesions on the cells, leading to tumour destruction are believed to be on the nuclear DNA (Rosenberg, 1977). However, tests conducted in vitro indicate a large number of possible modes of reaction of the nucleic acid and its constituents. Events subsequent to the primary attack that lead to anti-tumour activity are largely unknown. There is some evidence suggesting the involvement of the host's immune response in the anti-tumour activity (Rosenberg, 1977).

Cis-DDP has several advantages as an anti-tumour agent:

1. it exhibits marked anti-tumour activity
2. it is a broad spectrum drug that is active against drug-resistant as well as drug-sensitive tumours
3. it is active against both slow-growing and fast-growing tumours
4. it has some activity against tumours insensitive to S-phase inhibitors
5. it is useful against disseminated as well as solid tumours
1.7.4. **Cell cycle and combination chemotherapy.**

The combination of several anti-tumour agents in enhancing the efficacy of treatment of cancer is based mainly on their inhibition of different phases of the cell cycle.

The process of cell division involves several stages. Firstly, the daughter cells produced by a previous division undergo a period of cytoplasmic growth in which they increase in size and synthesize products needed for specialized cell function. This initial period of growth is called 'G\textsubscript{1}' and involves a rapid turnover of RNA which in turn directs the synthesis of proteins. Most tissue cells spend their lives in G\textsubscript{1} and cannot subsequently de-differentiate and divide.

A few cells, called stem cells or immature G\textsubscript{1} cells, retain the capacity to respond to a replicative stimulus. After appropriate initiating stimuli, such stem cells begin to synthesize products necessary for DNA synthesis (late G\textsubscript{1} phase). G\textsubscript{1} is therefore, a period of extremely variable length, ranging from a few hours to many years.

Thereafter, the cells enter the 'S' phase, a rather restricted period of intense DNA replication. During this period, the purine and pyrimidine bases are synthesized, converted to nucleotides and combined into new strands of DNA that are complimentary to the single components of the double helix. During the S phase many targets for chemotherapy are provided by the need of the cells to synthesize purine and pyrimidine bases, the appearance of enzymes concerned with biosynthesis, phosphorylation and polymerisation of bases and the
opening up of the double helix with uncovering of multiple reactive sites along the DNA chain (Brule et al., 1973). A cell that enters the S phase is committed to proceed through all the subsequent steps of the mitotic cycle.

Following the S phase, there is a rather brief period of metabolic consolidation of the cell; this phase is called 'G2'. It is a period during which general cytoplasmic proteins and possibly histones and other DNA associated proteins are synthesized.

The cell then enters the mitosis, or 'M' phase. In most cells, regardless of the tissue or even the species of origin, M phase lasts for about an hour.

Tumour cells also follow the same cycle of events. Three known factors that affect the rate of tumour growth are: (1) the rate of replication of proliferating cells (cell cycle time) (2) the proportion of total cell population that is proliferating (growth fraction) and (3) the rate of cell loss from the tumour (Steel et al., 1966). The rate of cell proliferation depends on cell type and is influenced by the availability of nutrients including oxygen.

Several authors have reviewed the mode of action of antineoplastic agents (Stock, 1975; Krakoff, 1977). Some antineoplastic agents are cell cycle dependent, while others are not. The site of action of some antineoplastic drugs in the cell cycle is shown in Fig. 1.10. The anti-tumour agent bleomycin, reacts with DNA either in the late S phase or G2 phase. Vinblastine, however, inhibits cell division
Fig. 1.10 Site of action of antineoplastic agents in the cell cycle. (Adapted from Clinical Oncology, International Union against cancer, 1978).
possibly in the late G$_2$ and the M phase. The combination of vinblastine with bleomycin would greatly enhance the anti-tumour effect of each drug. Moreover, the different biochemical properties of these drugs would enable the toxic side effects to be spread. The toxic side effects of bleomycin are seen in the lungs and skin (Umezawa, 1970). In fact, impairment of pulmonary function is the dose-limiting factor (Luna et al., 1972), but, it is exceptional, in that it does not depress the bone marrow. Vinblastine, however, is notably toxic to the bone marrow (Creasy, 1975). Thus a combination of these drugs would enable the use of the two drugs in sufficiently high concentration to produce a marked anti-tumour effect with minimal side effects. In contrast, cis-DDP is not cycle specific and its dose-limiting factor is toxicity to the kidney (Gottlieb and Drewinko, 1974). The use of cis-DDP in combination with vinblastine and bleomycin would be expected to achieve a greater degree of specificity and enhance the efficacy of treatment. Also, the emergence of resistance is likely to be delayed when a combination of drugs is used.

1.7.5. Adverse effects of chemotherapy.

An ideal chemotherapeutic agent should have the capacity to interfere with a metabolic pathway that is unique to the neoplastic cells and necessary for their survival, without affecting normal cells. In addition, the drug should have characteristics that enable it to reach all the neoplastic cells at a sufficiently high concentration. However, a consistent, exploitable biochemical difference between normal and malignant cells has not been found so far.
The use of cytotoxic drugs is based on the principle that neoplastic cells have a faster rate of multiplication when compared to normal cells. The cells of many kinds of cancer divide more slowly than their normal counterparts. In many instances, however, they spend a longer time in the actual process of cell division, so that neoplastic tissue often contains a greater percentage of cells undergoing division at any one time. This may confer a certain degree of selectivity in the mode of therapeutic action. However, the action of most cytotoxic drugs is not selective with respect to normal and neoplastic cells. When these drugs are employed in doses sufficient to inhibit the growth of tumour cells, they will also have an effect on normal cells possessing a high turnover rate, such as cells of the bone marrow, gastro-intestinal tract, hair follicles and germinal epithelium.

The magnitude of these effects will depend on the nature of the drug, dosage, duration of treatment, changes in rates of excretion or metabolism and also upon individual susceptibility. Generally speaking, better nourished patients are able to withstand higher doses of drugs (Shils, 1975). Nutrition also affects the rates at which drugs are metabolized (Basu and Dickerson, 1974) and hence their toxicity.
1.8 NUTRITIONAL PROBLEMS ASSOCIATED WITH CHEMOTHERAPY OF CANCER

Nearly all chemotherapeutic agents against cancer adversely affect dietary intake and weight loss is often observed during aggressive chemotherapy for cancer (Donaldson, 1977). A summary of the effects of different drugs used in chemotherapy is given in table 1.4. Bleomycin, vinblastine and many other drugs cause anorexia, nausea, vomiting and diarrhoea (Donaldson, 1977). The changes in the intestinal mucosa caused by antineoplastic agents are of great importance, as they may well result in malabsorption of nutrients. Intestinal mucosal changes associated with altered intestinal function have been observed with Vinca alkaloids (Dowling et al., 1970).

The treatment with cytotoxic drugs may induce or aggravate deficiencies of certain micronutrients such as vitamins. For example, thiamine deficiency observed in some patients with advanced cancer was exacerbated by treatment with 5-fluorouracil. Studies conducted in vivo and in vitro suggest that 5-fluorouracil causes a marked decrease in hepatic concentration of thiamine, with a concomitant decrease of coenzyme activity (Basu et al., 1979). Furthermore, the antitumour effect of thiosemicarbazone is enhanced by the administration of thiamine (Crim et al., 1967). Treatment with thiosemicarbazone (3-ethoxy 2-oxo butyraldehyde) an active antitumour agent against transplanted tumours led to a fall in the levels of thiamine and pantothenic acid in the livers of experimental animals. Supplementing tumour bearing rats with thiamine and
Table 1.4  Drugs used in chemotherapy and their effects (adapted from Hegedus and Pelham, 1975)

<table>
<thead>
<tr>
<th>Type</th>
<th>Drugs</th>
<th>Stomatitis</th>
<th>Anorexia</th>
<th>Vomiting</th>
<th>Nausea</th>
<th>Diarrhoea</th>
<th>Other effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkylating agents</td>
<td>Cyclophosphamide</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>Oral ulcerations</td>
</tr>
<tr>
<td></td>
<td>Melphalan</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nitrogen mustard</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>Metallic taste in mouth following injection, headache, fever</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>Actinomycin D</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>Oral ulcerations</td>
</tr>
<tr>
<td></td>
<td>Bleomycin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>Fever, pulmonary toxicity</td>
</tr>
<tr>
<td></td>
<td>Doxorubicin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>Cardiac toxicity, red urine</td>
</tr>
<tr>
<td>Antimetabolites</td>
<td>Cytarabine</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>Oral inflammation and/or ulcerations, abdominal pain</td>
</tr>
<tr>
<td></td>
<td>5-Fluorouracil</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>Oral ulcerations</td>
</tr>
<tr>
<td></td>
<td>Hydroxyurea</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>Constipation, ulceration of buccal mucosa, renal dysfunction</td>
</tr>
<tr>
<td></td>
<td>6-Mercaptopurine</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>Fever, liver dysfunction</td>
</tr>
<tr>
<td></td>
<td>Methotrexate</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>Hepatic toxicity, oral ulcerations, gingivitis</td>
</tr>
<tr>
<td>Vinca alkaloids</td>
<td>Vinblastine</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>Constipation, abdominal pain, glossitis, numbness in extremities</td>
</tr>
<tr>
<td></td>
<td>Vincristine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>Constipation, abdominal pain, numbness in extremities</td>
</tr>
<tr>
<td>Others</td>
<td>Nitrosoureas</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>Prolonged anorexia</td>
</tr>
</tbody>
</table>
pantothenic acid increased the activity of the antitumour agent (Petering et al., 1964).

The effect of antitumour agents on plasma vitamin A in cancer patients has not been investigated. However, there is some evidence to suggest that vitamin A enhances the action of some antitumour agents in animal models. For example, vitamin A potentiates the antitumour effect of cyclophosphamide on mammary adenocarcinoma of mice (Anton and Brandes, 1968). Also, vitamin A enhances the antitumour effects of BCNU (1,3 bis (2-chloroethyl) 1-nitroso urea) and to a lesser degree cyclophosphamide in murine L 1210 leukaemia (Cohen, 1972; Cohen and Carbone, 1972).

Furthermore, Basu et al. (1974c) have demonstrated an accentuation of the cell killing effects of chlorambucil by phenobarbital, caffeine and vitamin A. Moreover, a combination of vitamin A and 5-fluorouracil has been shown to cause marked regression of a skin tumour, keratoacanthoma, in rabbits. (Prutkin, 1973).
1.9 CONCLUSION AND PLAN OF PRESENT STUDIES

Considerable importance is now attached to the role of nutrition in the pathogenesis and prevention of cancer as evidenced by a number of symposia and reviews on the subject. (Nutrition and Cancer, 1976; Nutrition and Cancer, 1977; Nutrition and Cancer Therapy, 1977). In this connection vitamin A is of particular interest. Deficiency of vitamin A has been shown to give rise to metaplastic changes in epithelial tissues, specially in the respiratory, gastro-intestinal and urogenital tracts. These changes may later progress to neoplasia. Studies on experimental animals revealed that natural or synthetic retinoids have the ability to reverse such changes. Furthermore, retinoids in high doses prevent chemical carcinogenesis in epithelia such as that of the respiratory tract.

Epidemiological evidence also points to an association between vitamin A and epithelial cancer in man, particularly in the lung. Dietary intake of vitamin A has been shown to be negatively associated with lung cancer at all levels of cigarette smoking. A recent study has also revealed that high vitamin A intake reduces the relative risk of development of lung cancer in heavy smokers. In view of the fact that lung cancer is a disease which has a high incidence and a poor prognosis, it is of paramount importance to study the relationship between vitamin A and lung cancer.

The reports on the subject however, are conflicting and scanty. Therefore, it was considered necessary to study the serum vitamin A
status of lung cancer patients in a systematic manner in order to clarify the earlier findings.

The demonstration of an association between lung cancer and vitamin A deficiency does not necessarily reveal a role for the vitamin in the causation of the disease. Furthermore, other factors may contribute to the low levels of vitamin A. A study of some of the factors which may affect plasma vitamin A was undertaken in the hope that it would provide an insight into the mechanism by which the low circulating levels were produced.

It seemed to be of interest to study another type of cancer where the malignant disease arises secondary to a primary tumour elsewhere. Testicular teratoma is a highly malignant tumour which often metastasizes to the lung in the early stages. The vitamin A transport system was also studied in such patients.

Unlike primary lung cancer, disseminated testicular teratoma is very sensitive to chemotherapy. However, the aggressive chemotherapeutic treatment given to such patients often causes toxic side effects. Therefore, it was considered necessary to investigate the effects of these cytotoxic drugs on vitamin A. Moreover, no studies have so far been conducted on the effect of these drug regimens on vitamin A. The effect of combination chemotherapy on the plasma vitamin A transport system was followed in patients having metastatic testicular teratoma treated with the following drug regimens:
(1) vinblastine and bleomycin - Samuels regimen
(2) vinblastine, bleomycin and cis-diamine dichloro platinum (II) - Einhorn regimen.

The variation of vitamin A status was compared with that of other vitamins such as vitamin E, ascorbic acid, thiamine and pyridoxine.

A parallel investigation of the effects of these antitumour agents on the vitamin A status of normal healthy rats was also carried out.
CHAPTER TWO

VITAMIN A AND LUNG CANCER IN MAN
2.1 INTRODUCTION

A preliminary study of vitamin A in lung cancer patients revealed significantly lower concentrations of plasma vitamin A as compared to normal healthy subjects, or to patients having non-malignant lung diseases (Basu et al., 1976). However, Cohen et al. (1977) did not observe a significant difference between plasma concentrations of lung cancer patients and normal healthy subjects. These observations seemed to require clarification. Moreover, it seemed necessary to study possible factors which could account for the low circulating concentrations of vitamin A, since this might provide insight about its causation.

The concentration of vitamin A may be altered by several factors. Thus, it is reduced by a low dietary intake of vitamin A or its precursors. Blood levels are also reduced by malabsorption from the gut. Vitamin A is transported in the blood in association with a specific protein - retinol-binding protein, which in turn circulates as a biprotein complex with prealbumin (Kanai et al., 1968). It is possible, therefore, that low circulating levels of the vitamin could be due to a decreased availability of the carrier protein(s). Additionally, low blood levels might result from an increased need for vitamin A, due to the presence of the tumour.

Vitamin A deficiency as a result of decreased dietary intake is relatively rare in developed countries. However, in some conditions malabsorption of vitamin A occurs, but, in such conditions it seems likely that other fat-soluble vitamins would also be poorly absorbed.
Vitamin E, another fat-soluble vitamin is an important factor in the biological utilisation of vitamin A. The absorption of vitamin A is impaired in vitamin E-deficient rats and oral supplementation with vitamin E markedly increased the utilisation of vitamin A (Ames, 1969). In a recent study, oral administration of vitamin E to normal and vitamin A-deficient children resulted in a significant increase in plasma vitamin A concentration in both groups (Jagadeesan and Reddy, 1978).

Smith and co-workers (1973c) pointed out that zinc may be essential in the mobilisation of vitamin A from tissue stores. These workers found that zinc-deficient rats had low plasma concentrations of vitamin A, inspite of adequate liver stores of the vitamin and zinc therapy resulted in mobilisation of vitamin A from the liver. It is possible, therefore, that low plasma vitamin A levels in lung cancer patients might result from a deficiency of zinc.

Cristaelis et al. (1976) suggested that increased ACTH (adrenocorticotrophic hormone) secretion, followed by increased production of glucocorticoids in the adrenals, may occur in lung cancer patients, either due to the presence of tumour cells creating a state of stress, or to ectopic production by the lung tumour. These glucocorticoids may antagonise tissue vitamin A and in turn lead to low plasma levels of the vitamin.

Some of these factors were studied in a series of patients with newly diagnosed bronchial carcinoma. Age matched patients with non-malignant lung diseases and other non-malignant diseases served as controls.
2.2 PATIENTS

Twenty six newly diagnosed, histologically proven lung cancer patients (22 males, 4 females) admitted for treatment to Redhill General Hospital, East Surrey were studied. Their ages ranged from 46-82 years with a mean value of 64.7 years (Table 2.1). Ten patients had squamous cell carcinoma, five oat cell carcinoma, three adenocarcinoma and eight had undifferentiated carcinoma. These patients had not previously received surgery, radiotherapy or chemotherapy.

The results of these patients were compared with those of ten patients (7 males, 3 females) having non-malignant lung diseases, such as acute or chronic bronchitis, bronchiectasis (control group I). Their ages ranged from 47-74 years, with a mean value of 60.3 years (Table 2.2). The second control group (II) consisted of eleven patients (8 males, 3 females) having other non-malignant diseases such as ischaemic heart disease, hiatus hernia, myocardial infarction and cerebro-vascular incident. Their ages ranged from 48-75 years, with a mean value of 63.4 years (Table 2.2). The smoking habits of both lung cancer patients and controls were noted.

Overnight fasting blood samples were collected by venepuncture. Serum was separated within two hours of withdrawal of blood, and divided into aliquots and stored in sample tubes covered with foil at -40°C until analysed. The analyses for vitamins A and E and β-carotene were carried out within two weeks of collection of the samples.
Table 2.1. Clinical details of lung cancer patients.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sex</th>
<th>Age years</th>
<th>Diagnosis</th>
<th>Smoking habits</th>
</tr>
</thead>
<tbody>
<tr>
<td>P.D.</td>
<td>M</td>
<td>60</td>
<td>Squamous carcinoma</td>
<td>10/day</td>
</tr>
<tr>
<td>G.R.</td>
<td>M</td>
<td>81</td>
<td>Advanced undifferentiated carcinoma</td>
<td>12/day</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>stopped at 60yrs</td>
</tr>
<tr>
<td>B.P.</td>
<td>F</td>
<td>71</td>
<td>Undifferentiated carcinoma</td>
<td>20/day</td>
</tr>
<tr>
<td>E.B.</td>
<td>F</td>
<td>78</td>
<td>Squamous carcinoma</td>
<td></td>
</tr>
<tr>
<td>F.C.</td>
<td>M</td>
<td>82</td>
<td>Oat cell carcinoma</td>
<td>pipe till 1960</td>
</tr>
<tr>
<td>F.L.</td>
<td>M</td>
<td>71</td>
<td>Squamous cell carcinoma, family history of lung cancer</td>
<td>20-30/day</td>
</tr>
<tr>
<td>E.C</td>
<td>M</td>
<td>54</td>
<td>Undifferentiated carcinoma, inoperable</td>
<td>5/day</td>
</tr>
<tr>
<td>D.I.</td>
<td>M</td>
<td>63</td>
<td>Bronchoalveloar adeno carcinoma, operable</td>
<td>20/day</td>
</tr>
<tr>
<td>C.G.</td>
<td>M</td>
<td>62</td>
<td>Oat cell carcinoma, operable</td>
<td>20/day</td>
</tr>
<tr>
<td>F.V.</td>
<td>M</td>
<td>66</td>
<td>Squamous carcinoma</td>
<td>40/day</td>
</tr>
<tr>
<td>F.B.</td>
<td>M</td>
<td>78</td>
<td>Inoperable, undifferentiated carcinoma</td>
<td></td>
</tr>
<tr>
<td>S.G.</td>
<td>F</td>
<td>46</td>
<td>Undifferentiated carcinoma of bronchus</td>
<td>7/day</td>
</tr>
<tr>
<td>R.N.</td>
<td>M</td>
<td>46</td>
<td>Large cell, oat cell and undifferentiated carcinoma</td>
<td></td>
</tr>
<tr>
<td>W.R.S.</td>
<td>M</td>
<td>62</td>
<td>Poorly differentiated squamous carcinoma</td>
<td>1 oz/day</td>
</tr>
<tr>
<td>E.T.</td>
<td>F</td>
<td>66</td>
<td>Adenocarcinoma</td>
<td>10/day</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>stopped 6 yrs ago</td>
</tr>
<tr>
<td>J.E.M.</td>
<td>M</td>
<td>60</td>
<td>Undifferentiated carcinoma</td>
<td>10/day</td>
</tr>
<tr>
<td>J.S.</td>
<td>M</td>
<td>77</td>
<td>Poorly differentiated squamous carcinoma</td>
<td>Smoker</td>
</tr>
<tr>
<td>D.B.</td>
<td>M</td>
<td>64</td>
<td>Oat cell carcinoma</td>
<td>80/day</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>stopped in 1967</td>
</tr>
<tr>
<td>C.M.</td>
<td>M</td>
<td>64</td>
<td>Oat cell carcinoma</td>
<td>60-80/day</td>
</tr>
<tr>
<td>Name</td>
<td>Sex</td>
<td>Age (years)</td>
<td>Diagnosis</td>
<td>Smoking habits</td>
</tr>
<tr>
<td>-------</td>
<td>-----</td>
<td>-------------</td>
<td>----------------------------</td>
<td>----------------</td>
</tr>
<tr>
<td>D.W.</td>
<td>M</td>
<td>49</td>
<td>Squamous carcinoma</td>
<td>2 oz/week</td>
</tr>
<tr>
<td>W.P.</td>
<td>M</td>
<td>63</td>
<td>Undifferentiated carcinoma</td>
<td>50/day</td>
</tr>
<tr>
<td>R.P.</td>
<td>N</td>
<td>57</td>
<td>Squamous carcinoma</td>
<td>30/day</td>
</tr>
<tr>
<td>T.H.</td>
<td>M</td>
<td>68</td>
<td>Adenocarcinoma</td>
<td>10/day till 1974</td>
</tr>
<tr>
<td>J.R.</td>
<td>M</td>
<td>60</td>
<td>Squamous carcinoma</td>
<td>20/day</td>
</tr>
<tr>
<td>C.G.</td>
<td>M</td>
<td>65</td>
<td>Squamous carcinoma</td>
<td>10/day</td>
</tr>
<tr>
<td>A.H.</td>
<td>M</td>
<td>70</td>
<td>Undifferentiated carcinoma</td>
<td>10/day</td>
</tr>
</tbody>
</table>
### Table 2.2 Clinical details of controls.

**Control group I - non-malignant lung diseases.**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Diagnosis</th>
<th>Smoking habits</th>
</tr>
</thead>
<tbody>
<tr>
<td>M.C.</td>
<td>M</td>
<td>54</td>
<td>Bronchitis</td>
<td>20-40/day</td>
</tr>
<tr>
<td>G.F.</td>
<td>M</td>
<td>72</td>
<td>Bronchitis</td>
<td>6/day</td>
</tr>
<tr>
<td>V.F.O.</td>
<td>M</td>
<td>74</td>
<td>Bronchitis</td>
<td>10/day</td>
</tr>
<tr>
<td>L.H.</td>
<td>F</td>
<td>68</td>
<td>Bronchitis</td>
<td>20/day</td>
</tr>
<tr>
<td>L.D.</td>
<td>M</td>
<td>65</td>
<td>Chronic bronchitis</td>
<td>Smoker</td>
</tr>
<tr>
<td>H.S.</td>
<td>F</td>
<td>63</td>
<td>Bronchiectasis</td>
<td>Non smoker</td>
</tr>
<tr>
<td>E.E.</td>
<td>F</td>
<td>60</td>
<td>Cor pulmonale</td>
<td>30/day</td>
</tr>
<tr>
<td>P.L.</td>
<td>M</td>
<td>51</td>
<td>Chronic bronchitis</td>
<td>20/day stopped in 1975</td>
</tr>
<tr>
<td>J.S.</td>
<td>M</td>
<td>47</td>
<td>Acute chest infection</td>
<td>50/day</td>
</tr>
<tr>
<td>R.W.</td>
<td>M</td>
<td>49</td>
<td>Haemoptysis</td>
<td></td>
</tr>
</tbody>
</table>

**Control group II - other non-malignant diseases.**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Diagnosis</th>
<th>Smoking habits</th>
</tr>
</thead>
<tbody>
<tr>
<td>C.C.</td>
<td>M</td>
<td>72</td>
<td>Ischaemic heart disease</td>
<td>10/day</td>
</tr>
<tr>
<td>B.H.</td>
<td>F</td>
<td>48</td>
<td>Oesophageal stricture</td>
<td>Non smoker</td>
</tr>
<tr>
<td>J.H.</td>
<td>M</td>
<td>52</td>
<td>Myocardial infarction</td>
<td>Non smoker</td>
</tr>
<tr>
<td>M.H.</td>
<td>F</td>
<td>75</td>
<td>Myocardial infarction</td>
<td>Non smoker</td>
</tr>
<tr>
<td>J.E.K.</td>
<td>M</td>
<td>60</td>
<td>Myocardial infarction</td>
<td></td>
</tr>
<tr>
<td>H.W.</td>
<td>M</td>
<td>68</td>
<td>Cerebrovascular incident</td>
<td>Non smoker</td>
</tr>
<tr>
<td>H.O.</td>
<td>M</td>
<td>70</td>
<td>Cerebrovascular incident</td>
<td>Stopped 15 yrs ago</td>
</tr>
<tr>
<td>S.S.</td>
<td>M</td>
<td>63</td>
<td>Cardiac failure</td>
<td>10/day</td>
</tr>
<tr>
<td>J.W.</td>
<td>M</td>
<td>57</td>
<td>Glandular fever</td>
<td>5/day</td>
</tr>
<tr>
<td>R.S.</td>
<td>F</td>
<td>63</td>
<td>Hiatus hernia</td>
<td>Non smoker</td>
</tr>
<tr>
<td>R.H.</td>
<td>M</td>
<td>69</td>
<td>Hiatus hernia</td>
<td>20/day</td>
</tr>
</tbody>
</table>
Statistical analyses were carried out using an Olivetti P652 computerised calculator and the significance was determined using the students t-test.
2.3 ANALYTICAL METHODS

2.3.1. Determination of vitamins A and E in the serum.

Vitamins A and E were determined simultaneously by a modification of the fluorometric method of Hansen and Warwick (1969). They measured the fluorescence of vitamin A at an excitation wave length of 340nm and an emission wave length of 480nm. However, there is considerable interference from carotenoids at this wave length (Thompson et al., 1973; Steveninck and de Goeij, 1973). Steveninck and de Goeij (1973) suggested the measurement of fluorescence at an emission wave length of 550nm, where interference from carotenoids is virtually zero.

The fluorescence was accordingly measured at an emission wave length of 550nm. The intensity of fluorescence increased linearly with concentration (Fig. 2.1) and there was no interference from carotenoids.

Under the same conditions, vitamin E exhibits fluorescence at an excitation wave length of 295nm and an emission wave length of 340nm (Hansen and Warwick, 1969; Thompson et al., 1973). Vitamin E was determined simultaneously. The presence of vitamin A or other carotenoids has been shown not to interfere with the determination of vitamin E (Hansen and Warwick, 1969). The fluorescence of vitamin E increased linearly with concentration (Fig.2.2).

All-trans retinyl acetate (Sigma) in absolute ethanol was used as the standard for vitamin A. The absorbance of the working
standard solution (10\mu g/ml) was checked at 326nm using an SP-1800 spectrophotometer before use (E \textsubscript{1cm,ethanol}^1 = 1550; Merck Index, 1968). DL-\alpha-tocopherol (Sigma) in ethanol was used as the standard for vitamin E and the absorbance of the stock solution (100\mu g/ml) was checked at 294nm before use (E \textsubscript{1cm,ethanol}^1 = 71; Merck Index, 1968). All glassware used was soaked overnight in 10% nitric acid, washed several times with tap water and distilled water to remove any traces of detergent.

Aliquots (0.5ml) of serum were pipetted into 15ml Sovril tubes fitted with teflon-lined caps. The same volume of water or standard was used instead of serum in the blank and standard respectively. Distilled water (1ml) was added to each tube, mixed and 2ml of absolute ethanol was added slowly with mixing to precipitate proteins. Five millilitres of spectroscopic grade hexane (B.D.H) was then added; the tubes were capped and mixed for 30 seconds to ensure complete extraction of vitamin A from the aqueous ethanolic phase to the hexane layer. The tubes were then centrifuged at 1500 rpm for 10 min. in a Beckman J6 refrigerated centrifuge.

The upper hexane layer was separated and its fluorescence was measured using an MF3-Perkin Elmer fluorescence spectrophotometer. Recoveries of added vitamins A and E ranged from 95-100%.

2.3.2. Determination of serum \textit{\textbeta}-carotene.

A spectrophotometric method was employed in the determination of \textit{\textbeta}-carotene (Neeld and Pearson, 1963).
Fig. 2.1 Standard curve for the determination of vitamin A
Fig. 2.2 Standard curve for the determination of vitamin E
The proteins were precipitated from the serum by adding 96% ethanol and ß-carotene was extracted from the aqueous ethanol phase using petroleum ether (A.R). The optical density of the petroleum ether layer was measured at 450nm using a Cecil spectrophotometer. All-trans ß-carotene (Sigma) in petroleum ether was used to obtain a standard curve (Fig 2.3).

2.3.3. Determination of retinol-binding protein in the serum.

The single radial immunodiffusion technique (Mancini et al., 1965) was used in the determination of retinol-binding protein.

LC-partigen immunodiffusion plates and stabilised standard human serum were obtained from Behring Diagnostics, Hoechst (U.K) Limited. An aliquot (20μl) of the diluted test serum (1:4) or standard serum was placed in each well in the immunodiffusion plate. At least three different dilutions of standard serum were placed on each plate. The plates were closed and incubated for 48 hours at room temperature. The diameters of the precipitin rings were measured using a calibrated lens against a dark background. The concentrations of retinol-binding protein in the test sera were obtained from a standard curve of the square of the diameter of the precipitin ring against concentration (Fig. 2.4).

2.3.4. Determination of prealbumin in the serum.

The concentration of prealbumin in the serum was determined by single radial immunodiffusion technique (Mancini et al., 1965).
Fig. 2.3 Standard curve for the determination of β-carotene
Fig. 2.4 Standard curve for the determination of retinol-binding protein

Concentration of retinol-binding protein (mg/100ml)

Diameter 2 (mm²)
A gram of agarose (Mercia Brocades Limited) was mixed with 0.024 M barbitone buffer, pH 8.6 and heated gently with constant stirring, until the agarose had completely dissolved. An aliquot (10ml) of molten agarose was transferred to a large pyrex tube and placed in a water bath maintained at 55°C for 5-10 min. One hundred microlitres of rabbit antiserum against human prealbumin (Hoechst (U.K) Limited) was added to the molten agarose, mixed gently and allowed to stand at 55°C for 5 min. The molten agarose containing antiserum was poured uniformly on a warm glass plate (8.2 x 8.2cm) kept on a balanced table and allowed to set. Wells were cut in the agarose when it had set using a gel-puncher and a punching template. An aliquot (3μl) of diluted serum (1:4) or stabilised standard serum (Hoechst (U.K) Limited) in varying dilutions was placed in each well. The plates were incubated for 48 hours in moistened, air tight plastic boxes at room temperature.

After diffusion was complete, the gel was pressed to remove any precipitated proteins. It was then dried in a current of warm air and stained by immersing the plate in Coomassie brilliant blue stainer for 2-3 min. The background colour was washed off by immersing the plate in ethanol-acetic acid destainer. The plate was dried in a current of warm air and the diameters of precipitin rings were measured using a calibrated lens. Prealbumin concentrations of test sera were obtained from a plot of the square of the diameter of the precipitin rings against concentration of stabilised sera (Fig. 2.5).
Fig. 2.5. Standard curve for the determination of prealbumin

Concentration of prealbumin (mg/100ml) vs. Diameter 2 (mm²)
2.3.5. **Determination of proteins in the serum.**

Total proteins were determined colorimetrically using the Biuret reagent (Sigma).

Total globulins were measured by the intensity of the colour given with the glyoxylic acid reagent (Sigma).

The albumin concentration was obtained from the difference between total protein and total globulin concentrations. Standard curves were plotted (Figs 2.6 and 2.7) using a standard protein solution (Sigma) containing human albumin (5g/100ml) and human globulin (3g/100ml).

2.3.6. **Determination of 11-hydroxycorticosteroid levels in the serum.**

The glucocorticoids in the serum were measured as 11-hydroxycorticosteroids. Mattingly's method (1962) was employed for the determination of 11-hydroxycorticosteroids. Corticosteroids were extracted from the serum with methylene chloride and the fluorescence obtained by treatment with ethanol-sulphuric acid reagent was measured.

To an aliquot (0.2ml) of serum in a 10ml Sovril tube, 1ml of water and 4ml chloride (B.D.H, Spectroscopic grade) was added. Corticosteroids were extracted into methylene chloride by gentle mixing in a rotatory mixer for 20 minutes. A reagent blank and a standard containing water and cortisol (Sigma) respectively were carried through the procedure. The tubes were centrifuged and the top aqueous layer was removed by suction.
Fig. 2.6 Standard curve for the determination of total protein

Fig. 2.7 Standard curve for the determination of globulins
An aliquot (3ml) of the methylene chloride extract was transferred to a 10ml graduated tube. The fluorescence of six extracts of serum, blank and standard, were carried through the procedure on each occasion. At zero time, the fluorescence reagent (containing 7 volumes of sulphuric acid and 3 volumes of ethanol) was added to the blank and mixed vigorously for 20 seconds. This procedure was repeated by adding the fluorescence reagent at one minute intervals. The methylene chloride layer was sucked off from each tube in turn, starting with the blank. The fluorescence of each solution was measured at exactly 15 minutes after mixing the fluorescence reagent, using an MF3-Perkin Elmer fluorescence spectrophotometer, at an excitation wave length of 475nm and an emission wave length of 530nm. The standard curve obtained is shown in Fig. 2.8.

2.3.7. **Determination of zinc and copper in the serum.**

Zinc and copper concentrations in the serum were measured by atomic absorption spectrophotometry. Serum was diluted 1 in 5 with distilled water to avoid interference of the atomic absorption signal by the serum matrix. Zinc acetate (0.1mM) and cupric chloride (0.1mM) were used as the respective standards for zinc and copper and standard curves were plotted (Figs. 2.9 and 2.10). The following settings were used on IL 353 atomic absorption spectrophotometer:

<table>
<thead>
<tr>
<th></th>
<th>Zinc</th>
<th>Copper</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wave length</td>
<td>213.9nm</td>
<td>324.7nm</td>
</tr>
<tr>
<td>Slit width</td>
<td>320µm</td>
<td>320µm</td>
</tr>
<tr>
<td>Lamp current</td>
<td>5 mA</td>
<td>5 mA</td>
</tr>
<tr>
<td>Voltage</td>
<td>530 V</td>
<td>530 V</td>
</tr>
</tbody>
</table>
Fig. 2.8 Standard curve for the determination of 11-hydroxy corticosteroids
Fig 2.9 Standard curve for the determination of zinc

Concentration of zinc (μmoles/L)

Fig. 2.10 Standard curve for the determination of copper

Concentration of copper (μmoles/L)
2.4 RESULTS

The vitamin A concentration in the serum of lung cancer patients was significantly (p < 0.01) lower (Table 2.3) than that of control group I (non-malignant lung diseases) and control group II (other non-malignant diseases). The concentrations of vitamins A and E and β-carotene were similar in the two control groups. The β-carotene concentration tended to be somewhat lower in lung cancer patients (105 ± 9.3 µg/100ml) as compared to both control groups, but the difference was not significant (Table 2.3). The vitamin E concentration in the serum was similar in lung cancer patients and both control groups (Table 2.3).

Only two lung cancer patients had vitamin A values above the means of the two control groups (Fig. 2.11). Patients having other non-malignant diseases (control group II) had the highest mean value for vitamin A (61.9 ± 2.2 µg/100ml), while patients with non-malignant lung diseases (control group I) had a mean vitamin A concentration of 58.3 ± 1.5 µg/100ml. Four patients with advanced malignant disease had a vitamin A concentration of less than 30µg/100ml. Patients with squamous cell carcinoma (mean value - 46.4 µg/100ml) and undifferentiated carcinoma (mean value - 43.4 µg/100ml) had slightly lower mean vitamin A levels than those with oat cell carcinoma (50.5 µg/100ml), but, the difference was not significant (Fig. 2.11).

A significantly (p < 0.001) lower concentration of retinol-binding protein was also observed in the serum of lung cancer patients
Table 2.3. Vitamin A, β-carotene and vitamin E concentrations in the serum of lung cancer patients and controls.

Values are means ± SEM for the number of patients shown in parenthesis

<table>
<thead>
<tr>
<th></th>
<th>Vitamin A (µg/100ml)</th>
<th>β-carotene (µg/100ml)</th>
<th>Vitamin E (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung cancer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(26)</td>
<td>46.9 ± 2.4**†††</td>
<td>105 ± 9.3</td>
<td>9.0 ± 0.6</td>
</tr>
<tr>
<td>Control groups:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(I) non-malignant lung</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>diseases (10)</td>
<td>58.3 ± 1.6</td>
<td>120 ± 9.8</td>
<td>9.0 ± 0.6</td>
</tr>
<tr>
<td>(II) other non-malignant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>diseases (11)</td>
<td>61.9 ± 2.2</td>
<td>130 ± 8.9</td>
<td>9.6 ± 0.5</td>
</tr>
</tbody>
</table>

** Significantly different from I (p < 0.01)

††† Significantly different from II (p < 0.001)
Fig 2.11 Vitamin A concentrations in the serum of lung cancer patients and controls
as compared to both control groups. (Fig. 2.12). Lung cancer patients had a mean retinol-binding protein concentration of $4.1 \pm 0.23$ mg/100ml, while patients having non-malignant lung diseases (control group I) had a mean retinol-binding protein concentration of $5.38 \pm 0.15$ mg/100ml and those having other non-malignant diseases (control group II) had a mean value of $5.47 \pm 0.27$ mg/100ml.

Furthermore, serum vitamin A showed a highly significant positive correlation with retinol-binding protein ($r = 0.86$, p < 0.001) in lung cancer patients, but not in the control groups (Fig. 2.13).

The concentration of prealbumin in the serum of lung cancer patients was not significantly different from that of either of the two control groups (Fig. 2.14). Lung cancer patients had a mean prealbumin concentration of $20.8 \pm 1.7$ mg/100ml, as compared to control group I, which had a mean prealbumin level of $23.9 \pm 1.6$ mg/100ml, and control group II with a mean value of $25.0 \pm 1.5$ mg/100ml.

However, four patients who had very low values for vitamin A, had low values for retinol-binding protein and prealbumin as well. Three of these four patients (G.R., A.H. and F.C.) died within a short period of diagnosis. The lung cancer patient with the highest value of vitamin A (F.L.) had a family history of lung cancer.

The mean concentration of 11-hydroxycorticosteroids in lung cancer patients was not significantly different from that of the
Fig. 2.12 Retinol-binding protein concentrations in the serum of lung cancer patients and controls

- FL: 6.9, 6.6
- E.B
- G.R
- A.H
- F.C

Control group I: Non-malignant lung diseases
Control group II: Other non-malignant diseases

*** Significantly different from both control groups (p < 0.001)
Fig. 2.13 Relationship of vitamin A to retinol-binding protein in the serum of lung cancer patients and controls.

For lung cancer patients

\[ r = 0.86, p < 0.001 \]

\[ y = 10.0 + 8.97x \]

Controls N.S.
Fig 2.14 Prealbumin concentrations in the serum of lung cancer patients and controls
two control groups (Table 2.4).

Lung cancer patients had lower serum zinc levels when compared to both control groups and the difference was significant \((p < 0.05)\) when lung cancer patients were compared with control group II (Table 2.4). The mean concentration of copper in the serum of lung cancer patients was higher \((p < 0.05)\) than that of control group I (Table 2.4). The differences between the concentrations in the cancer patients and those in the other control group were in the same direction, but not significant. As a result of these differences, the zinc : copper ratio in cancer patients was significantly \((p < 0.01)\) lower than in either of the two control groups (Table 2.4).

The serum zinc concentrations showed a significant positive correlation \((r = 0.55, p < 0.01)\) with the concentration of serum vitamin A (Fig. 2.15). A highly significant positive correlation \((r = 0.55, p < 0.01)\) also existed between zinc and retinol-binding protein in the serum (Fig. 2.16).

The total protein, albumin and globulin levels were similar in the lung cancer patients and control groups (Table 2.5).
Table 2.4. Concentrations of zinc, copper and 11-hydroxycorticosteroids in the serum of lung cancer patients and controls.

Values are means ± SEM for the number of patients shown in parenthesis.

<table>
<thead>
<tr>
<th></th>
<th>Zinc (μM/L)</th>
<th>Copper (μM/L)</th>
<th>Zinc : Copper ratio</th>
<th>11-hydroxy corticosteroids (μg/100ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lunc cancer</td>
<td>(26) 12.8 ± 0.7†</td>
<td>23.9 ± 1.1*</td>
<td>0.52 ± 0.04††</td>
<td>16.8 ± 1.0</td>
</tr>
<tr>
<td>Control groups:-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(I) Non-malignant</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lung diseases</td>
<td>(10) 14.3 ± 1.3</td>
<td>19.9 ± 0.9</td>
<td>0.72 ± 0.07</td>
<td>14.5 ± 1.18</td>
</tr>
<tr>
<td>(II) Other non-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>malignant diseases</td>
<td>(11) 16.4 ± 2.0</td>
<td>21.4 ± 1.4</td>
<td>0.73 ± 0.05</td>
<td>15.9 ± 0.6</td>
</tr>
</tbody>
</table>

* Significantly different from (I) (p < 0.05, **p < 0.01)
† Significantly different from (II) (†p < 0.05, ††p < 0.01)
Fig. 2.15  Relationship between zinc and vitamin A concentrations in the serum of lung cancer patients and controls

- Lung cancer patients
- Control group I (non-malignant lung diseases)
- Control group II (other non-malignant diseases)

For lung cancer patients

\( r = 0.552, p < 0.01 \)
\( y = 5.76 + 0.12 x \)

Controls N.S.
Fig. 2.16  Relationship between zinc and retinol-binding protein concentrations in the serum of lung cancer patients and controls

- Lung cancer patients
- Control group I (non-malignant lung diseases)
- Control group II (other non-malignant diseases)

For lung cancer patients
\[ r = 0.554, p < 0.01 \]
\[ y = 7.126 + 1.29x \]
Controls N.S.
Table 2.5. Serum protein concentrations in lung cancer patients and controls.

Values are means ± SEM for the number of patients shown in parenthesis

<table>
<thead>
<tr>
<th></th>
<th>Total protein (g/L)</th>
<th>Albumin (g/L)</th>
<th>Total globulin (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lung cancer</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(26)</td>
<td>66.8 ± 1.1</td>
<td>39.0 ± 1.5</td>
<td>28.8 ± 1.4</td>
</tr>
<tr>
<td><strong>Control groups:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(I) Non-malignant lung</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>diseases (10)</td>
<td>66.2 ± 1.2</td>
<td>42.0 ± 1.7</td>
<td>24.2 ± 1.0</td>
</tr>
<tr>
<td>(II) Other non-malignant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>diseases (11)</td>
<td>68.0 ± 1.2</td>
<td>42.3 ± 1.4</td>
<td>25.6 ± 1.4</td>
</tr>
</tbody>
</table>
2.5. DISCUSSION

Significantly lower vitamin A concentrations were found in the serum of lung cancer patients, as compared to control groups having either non-malignant lung diseases or other non-malignant diseases. These findings are in agreement with the earlier observation of Basu et al. (1976). However, the present findings differ from those of Cohen and co-workers (1977), who studied the dietary vitamin A intake and serum vitamin A in non-resectable lung cancer patients. The mean value of vitamin A in the control groups of the present study is 60μg/100ml, which is somewhat higher than the mean control value of 50μg/100ml, quoted by Cohen et al. Moreover, these workers quoted a value for a control population. They did not, in fact, determine the control value in an age-matched population. The mean values in their patients tended to be somewhat higher than the values obtained in the present study.

The finding of lower concentrations of vitamin A in the serum of these patients may suggest a possible role of this vitamin in the aetiology of lung cancer, as has been noted in experimental animals (Nettesheim and Williams, 1976; Nutr. Rev., 1979). However, the importance of vitamin A as an aetiological factor in human lung cancer is not at present clear.

Cigarette smoking is an important contributory factor in the causation of lung cancer (Doll and Peto, 1976). It is possible that the carcinogenic polycyclic hydrocarbons present in cigarette smoke may affect the plasma vitamin A by depleting body stores of this vitamin. In fact, an early study showed that large doses of intra-peritoneally administered 3,4 benzo(a)pyrene and
3-methyl cholangthrene results in a marked reduction in hepatic vitamin A in the rat. (Carruthers, 1942). Recently Mettlin and co-workers (1979) have reported that high dietary vitamin A intake reduces the risk of development of lung cancer in smokers, particularly in heavy smokers. However, the present study did not reveal an association between serum vitamin A and smoking habits in lung cancer patients. This may be due to the difficulties encountered in obtaining accurate smoking histories from patients, in view of the variability of smoking habits over a period of time.

The concentration of 11-hydroxycorticosteroids in the serum of lung cancer patients was not significantly different from that of the controls. Thus there was no evidence in these patients to support the suggestion of Cristaelis and co-workers (1976) that elevated glucocorticoids were responsible for the low vitamin A levels observed in lung cancer patients. However, glucocorticoids may be an important contributory factor in depressing vitamin A in patients having lung tumours with ectopic production of ACTH. This is frequently observed in oat-cell carcinoma of the lung (Omenn and Wilkins, 1970; Nathanson and Hall, 1974). Only a few patients with oat cell carcinoma were included in the present study. The effect of corticosteroids (glucocorticoids) on vitamin A was studied experimentally in rats and the results are presented in Chapter Three.

Serum vitamin A levels may be affected by impaired fat absorption. However the serum concentration of vitamin E, another fat-soluble vitamin was unaltered in lung cancer patients. Also, \( \beta \)-carotene levels in the serum of lung cancer patients were not
significantly different from that of controls. Therefore, it is unlikely that serum vitamin A is decreased due to malabsorption of fat. Vitamin E is also important in the absorption and biological utilisation of vitamin A (Ames, 1969; Jagadeesan and Reddy, 1978). The lack of correlation between serum vitamins A and E suggests that decreased vitamin A is not a manifestation of vitamin E deficiency.

Vitamin A is transported to the target tissues bound to retinol-binding protein (Kanai et al., 1968). The observation of markedly decreased serum retinol-binding protein along with low serum vitamin A raises the interesting possibility that decreased vitamin A may be the result of a lower concentration of carrier protein. Furthermore, a highly significant correlation between serum vitamin A and retinol-binding protein was observed in lung cancer patients, but not in controls. Thus, it is possible that the delivery of vitamin A to the tissues is determined by the availability of the carrier protein in lung cancer patients.

Vitamin A bound to retinol-binding protein normally circulates as a biprotein complex with prealbumin (Raz et al., 1970; Peterson, 1971a). Prealbumin is also involved in the transport of thyroxine, but the two mechanisms are not inter-dependent (Raz and Goodman, 1969). The observation of similar values for the concentration of prealbumin in both lung cancer patients and controls, suggests that decreased circulating vitamin A and retinol-binding protein did not result from a deficiency of prealbumin.

The concentration of circulating retinol-binding protein and
prealbumin is very sensitive to the nutritional status of the individual (Ingenbleek et al., 1972; Ingenbleek et al., 1975a). Several workers have demonstrated a decrease in vitamin A and retinol-binding protein in children with protein-calorie malnutrition (Smith et al., 1973b; Ingenbleek et al., 1975b). Moreover, supplying calories and protein without supplemental vitamin A resulted in a clinical cure and a significant rise in vitamin A, retinol-binding protein and prealbumin (Smith et al., 1973b).

Malnutrition is often seen in patients with advanced malignant disease (Theologides, 1977; Leading article, British Medical Journal, 1979), although it is less common in malignant disease of the lung (Dickerson and Basu, 1978). Therefore, it is possible that the decreased vitamin A and retinol-binding protein was a manifestation of generalized nutritional deficiency. However, the observation of similar values for prealbumin in lung cancer patients and controls excludes this possibility. This is further supported by the fact that total protein and albumin concentrations were similar in lung cancer patients and controls. These results suggest that the observed low values are a specific effect on vitamin A and retinol-binding protein in lung cancer patients rather than a generalized nutritional deficiency.

Smith and co-workers (1974) suggested that zinc may be involved in the mobilisation of vitamin A from the liver. Zinc-deficient rats had low plasma vitamin A levels, while accumulating vitamin A in the liver (Smith et al., 1973c). Moreover, a markedly lower concentration of plasma retinol-binding protein has been reported in
zinc-deficient rats, as compared to zinc-sufficient rats fed ad libitum or pair-fed with zinc-deficient rats (Smith et al., 1974). Furthermore, the liver concentration of retinol-binding protein in zinc-deficient rats was only 55 - 60% of that of ad libitum or pair-fed controls. This data suggests that zinc deficiency probably interferes with the synthesis of retinol-binding protein. Repletion of zinc-deficient animals restored the plasma vitamin A concentration to values within the normal range, after three days of treatment (Brown et al., 1976). Mobilisation of vitamin A by intra-peritoneal administration of large doses of zinc sulphate has also been observed in normal male weanling rats (Ette et al., 1979).

In the present study, low vitamin A levels were associated with subnormal serum zinc levels. Other workers have also observed decreased blood and plasma zinc concentrations in patients with lung cancer (Davies et al., 1968; Morgan, 1970; Davies, 1972). The observed elevation of serum copper concentrations could have been due to an increase in the concentration of the carrier protein ceruloplasmin. Evidence in support of this suggestion was obtained by Mateo et al. (1979) who reported elevated serum copper levels which were significantly correlated with elevated serum ceruloplasmin levels. The serum zinc levels were significantly correlated with both vitamin A and retinol-binding protein in the serum. Therefore, it is possible that the decreased zinc in some way contributed to the decrease in circulating retinol-binding protein and vitamin A. Deficiency of zinc associated with decreased vitamin A, has also been observed in the plasma of patients with alcoholic cirrhosis (Smith et al., 1975b) and cystic fibrosis (Jacob and Sandstead, 1978). Michaelsson et al. (1977) have also observed significantly lower
concentrations of zinc and retinol-binding protein in the serum of male subjects with severe acne.

Zinc plays an important role in the synthesis of nucleic acids and proteins and is a constituent of thymidine kinase and DNA-dependent DNA and RNA polymerases (Vallee, 1977). Zinc is also required in certain steps of the cell multiplication cycle (Riordan, 1976). Thus, it is not surprising that zinc deprivation inhibits the growth of Walker-256 carcinosarcoma (De Wys et al., 1970), some leukaemias and Lewis lung carcinoma (Pories et al., 1978) in mice, and results in increased survival. Furthermore, human malignant lung and breast tissues have been found to contain significantly higher concentrations of zinc when compared to the normal non-cancerous tissue (Mulay et al., 1971; Schwartz et al., 1974). Therefore, it is tempting to suggest that the increased requirement for zinc during growth of the tumour may contribute at least in part to the decrease in circulating retinol-binding protein and vitamin A.
CHAPTER THREE

EFFECT OF CORTICOSTERONE ON THE

VITAMIN A STATUS OF RATS
3.1 INTRODUCTION

Increased ACTH secretion with excessive glucocorticoid production has been observed in some lung tumours, particularly the oat cell type (Marks, 1961; Nathanson and Hall, 1974). Adrenal hypertrophy with excessive secretion of glucocorticoids has also been observed in animals subjected to various types of stress, such as skin grafts, inoculation with tumour cells or physical stress (Seifter et al., 1976). Experimental studies have revealed that physical stress by partial body casting precipitates frank vitamin A deficiency in rats on a marginal intake of the vitamin (Seifter et al., 1973a). It is possible that stress-induced secretion of glucocorticoids antagonise tissue vitamin A by favouring its elimination from the body. Thus, an earlier study showed that administration of cortisone to rats over a long period resulted in loss of vitamin A from the liver and kidney (Clark and Colburn, 1955).

A relationship between vitamin A metabolism and corticosteroids (glucocorticoids) is further indicated by the fact that the vitamin antagonises the immune-suppressive effects of hydrocortisone in mice (Cohen and Cohen, 1973). Moreover, it prevents the development of steroid-induced ulcers in experimental animals (Hutcher et al., 1971) and reverses the inhibition of wound healing caused by glucocorticoids (Stephens et al., 1971; Ehrlich et al., 1973).

The present study was undertaken to ascertain if exogenous glucocorticoids antagonise vitamin A by lowering its tissue concentration. The effect of corticosterone on the levels of vitamin A in the plasma and various tissues of normal healthy rats
was studied. The plasma levels of vitamin E, another fat-soluble vitamin was also studied to determine whether the effect was specific to vitamin A.
3.2 EXPERIMENTAL PROTOCOL

Adult male Wistar-Albino rats weighing approximately 200g were used and maintained on a stock pellet diet (Spiller) and water ad libitum. The animals were kept in individual cages for two days prior to treatment to acclimatise them to their environment.

3.2.1. Effect of corticosterone treatment for different intervals of time.

The animals were treated with corticosterone (25mg/kg body weight) subcutaneously, twice daily and killed at 0, 1, 3 and 4 days following treatment. Blood was collected by cardiac puncture into heparinised tubes. The liver, thymus and adrenal glands were taken from each group of 5 animals. The separated plasma and weighed tissues were stored at -40°C until analysed.

3.2.2. Effect of treatment with corticosterone and retinol for one week.

Twenty-four rats were divided into 4 groups. Group A was given propylene glycol subcutaneously, twice daily and corn oil intraperitoneally, once daily and served as controls. Group B was treated with corticosterone in propylene glycol (15mg/kg body weight, subcutaneously twice daily) and corn oil intraperitoneally. Group C was treated with retinol in corn oil (3000 IU per animal, intraperitoneally, once daily) in addition to corticosterone. Group D was given retinol (3000 IU, intraperitoneally, once daily) and
propylene glycol.

All animals were treated for 7 successive days and killed on the 8th day and blood and organs were collected as described in the previous study (3.2.1).
3.3 ANALYTICAL METHODS

The concentrations of vitamins A and E in the plasma were determined by the fluorometric method described earlier for the serum (Chapter Two).

3.3.1 Determination of vitamin A in tissues.

The vitamin A content of tissues was determined fluorometrically (Thompson et al., 1971). A weighed amount of tissue (0.1 - 0.5g) was saponified by heating in a boiling water bath for 15 minutes with 60% aqueous potassium hydroxide (0.5ml) in the presence of 1% ethanolic pyrogallol (1 ml). The tubes were then cooled and 1 ml of water was added. The unsaponifiable matter was extracted with hexane and its fluorescence was measured as described for the serum (Chapter Two).

In the determination of vitamin A content of the liver, about 0.1 g of tissue was used and the extraction was carried out with two 5 ml aliquots of hexane. The fluorescence of the pooled extracts was measured. The standard curve is shown in Fig. 3.1.
Fig. 3.1 Standard curve for the determination of tissue vitamin A
3.4 RESULTS

3.4.1 Effect of corticosterone treatment for different intervals of time.

The body weight of the animals treated with corticosterone (25mg/kg, twice daily) remained more or less unchanged after the 4 day treatment (Table 3.1). The restriction of body weight gain was not due to decreased food intake. The absolute liver weights of steroid-treated animals increased slightly on the first day and decreased to its normal value on further treatment (Table 3.1). The liver weight relative to body weight showed a similar variation, increasing significantly (p < 0.05) on the first day and returning to normal values on further treatment (Fig. 3.2).

Both the absolute and relative weights of adrenals decreased gradually and the decrease was significant (p < 0.05) only after three days of corticosterone administration. Significant (p < 0.01) thymic involution occurred even after treatment with corticosterone for one day, and further treatment resulted in a gradual decrease in weight of the thymus (Table 3.1). The thymus weight relative to body weight was also markedly decreased on steroid administration (Fig. 3.2).

The concentrations of plasma vitamins A and E in animals receiving corticosterone for various periods are shown in Table 3.2. The plasma concentration of vitamin A decreased significantly (p < 0.05) after 3 days of treatment, while the plasma vitamin E levels fell significantly (p < 0.05) only after 4 days. The liver vitamin A
Table 3.1. Effect of treatment with corticosterone for different intervals of time on body and tissue weights of rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Control day 0</th>
<th>day 1</th>
<th>day 3</th>
<th>day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight gain (g)</td>
<td>-</td>
<td>4.2 ± 0.73</td>
<td>3.2 ± 1.1</td>
<td>1.8 ± 0.6</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>10.8 ± 0.68</td>
<td>12.0 ± 0.16</td>
<td>10.2 ± 0.34</td>
<td>10.2 ± 0.46</td>
</tr>
<tr>
<td>Thymus weight (g)</td>
<td>0.67 ± 0.052</td>
<td>0.30 ± 0.027</td>
<td>0.13 ± 0.010</td>
<td>0.09 ± 0.012</td>
</tr>
<tr>
<td>Weight of adrenals (mg)</td>
<td>49.7 ± 7.2</td>
<td>42.7 ± 2.7</td>
<td>24.2 ± 2.9</td>
<td>*25.5 ± 3.6</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SEM of 5 rats.

* Significantly different from control (* p < 0.05, ** p < 0.01, *** p < 0.001).
Fig. 3.2. Effect of corticosterone treatment on organ weight relative to body weight (△ liver, □ adrenal glands, ◦ thymus).

Each value is the mean ± SEM for 5 rats.

* Significantly different from control (* p < 0.05, ** p < 0.01, *** p < 0.001)
concentration was more sensitive to corticosterone treatment. decreasing significantly (p < 0.05) even after one day (Table 3.2). The total hepatic content of vitamin A also showed a gradual decrease on steroid administration.

The amount of vitamin A in the adrenal glands showed a gradual fall, and it was significant from day 3 of treatment (Fig. 3.3). The vitamin A content of the thymus, however, decreased significantly (p < 0.01) even after administration of corticosterone for one day. Further treatment resulted in a gradual depletion of vitamin A from the thymus (Fig. 3.3).

3.4.2 Effect of treatment with corticosterone and retinol for one week.

Treatment with corticosterone (15mg/kg, twice daily) for one week caused a marked reduction in body weight gain (Table 3.3) which remained unchanged even when the steroid was administered in combination with retinol (3000 IU). Corticosterone did not affect and corticosterone the weight of the liver, while retinol caused a slight increase in liver weight relative to body weight (Table 3.3). In the corticosterone-treated animals, there was a decrease of about 70% in absolute thymus weight when compared with the control animals (Table 3.3). Like the liver, the absolute weight of the thymus, remained unchanged even when the steroid was administered concomitantly with retinol. Also, it was noted that the animals treated with retinol alone had a thymus weight similar to the controls. The thymus weight relative to body weight was slightly elevated in retinol-treated animals, but the increase was not significant. The weight of adrenal glands was also decreased with corticosterone and simultaneous
Table 3.2.  Effect of corticosterone treatment for different intervals of time.

<table>
<thead>
<tr>
<th>Group</th>
<th>Control day 0</th>
<th>day 1</th>
<th>day 3</th>
<th>day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma vitamin A (µg/100ml)</td>
<td>56 ± 4.6</td>
<td>56 ± 5.8</td>
<td>44 ± 1.7*</td>
<td>44 ± 1.6*</td>
</tr>
<tr>
<td>Plasma vitamin E (µg/ml)</td>
<td>7.4 ± 0.58</td>
<td>7.1 ± 0.54</td>
<td>6.0 ± 0.41</td>
<td>6.1 ± 0.09*</td>
</tr>
<tr>
<td>Liver vitamin A concentration (µg/g)</td>
<td>137 ± 4</td>
<td>108 ± 6  *</td>
<td>82 ± 15  *</td>
<td>118 ± 5</td>
</tr>
<tr>
<td>Total liver vitamin A (mg)</td>
<td>1.47 ± 0.06</td>
<td>1.29 ± 0.07</td>
<td>0.83 ± 0.16**</td>
<td>1.19 ± 0.04**</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SEM for 5 rats.

* Significantly different from control (* p < 0.05, ** p < 0.01, *** p < 0.001)
Fig. 3.3  Effect of corticosterone treatment on vitamin A content of the thymus (○) and adrenal glands (■).

Each value is the mean ± SEM for 5 rats.

* Significantly different from control. (* p < 0.05, ** p < 0.01, *** p < 0.001)
Table 3.3  Effect of treatment with corticosterone and retinol for one week on body and tissue weights of rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>Corticosterone</th>
<th>Corticosterone + retinol</th>
<th>Retinol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight gain (g)</td>
<td>47 ± 2.6</td>
<td>29 ± 2.6 ***</td>
<td>29 ± 2.5 ***</td>
<td>48 ± 3.5</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>12.5 ± 0.47</td>
<td>11.9 ± 0.73</td>
<td>12.3 ± 0.6</td>
<td>12.3 ± 0.93</td>
</tr>
<tr>
<td>Liver weight (%)</td>
<td>4.65 ± 0.11</td>
<td>5.03 ± 0.10</td>
<td>5.25 ± 0.10 *</td>
<td>4.79 ± 0.23</td>
</tr>
<tr>
<td>Thymus weight (g)</td>
<td>0.58 ± 0.04</td>
<td>0.17 ± 0.02 ***</td>
<td>0.21 ± 0.02 ***</td>
<td>0.54 ± 0.03</td>
</tr>
<tr>
<td>Thymus weight (%)</td>
<td>0.198 ± 0.009</td>
<td>0.075 ± 0.011</td>
<td>0.088 ± 0.008</td>
<td>0.212 ± 0.007</td>
</tr>
<tr>
<td>Weight of Adrenals (mg)</td>
<td>41.8 ± 3.26</td>
<td>24.7 ± 2.29 **</td>
<td>18.7 ± 1.42 ***</td>
<td>37.6 ± 2.66</td>
</tr>
<tr>
<td>Adrenal weight (%)</td>
<td>0.014 ± 0.001</td>
<td>0.011 ± 0.001</td>
<td>0.008 ± 0.001</td>
<td>0.015 ± 0.001</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SEM of 6 rats.

* Significantly different from control (* p < 0.05, ** p < 0.01, *** p < 0.001)
treatment with retinol did not alter the weight loss.

The plasma concentration of vitamin A fell significantly (p < 0.01) in animals treated with corticosterone alone, but not in those treated with the steroid in combination with retinol (Table 3.4). The vitamin E concentration also fell (p < 0.05) on steroid-treatment, but to a lesser extent. The plasma levels of vitamin E were restored to control values by concomitant retinol treatment. The vitamin A contents of the liver and adrenal glands fell significantly on corticosterone treatment and rose to control values in animals treated with corticosterone in combination with retinol. Retinol treatment alone increased the vitamin A contents of the liver and adrenal glands to values much higher than the controls.

The loss of vitamin A resulting from the administration of corticosterone was most marked (p < 0.001) in the thymus. (Table 3.4). Concomitant administration of retinol increased the vitamin A content of the thymus to values significantly (p < 0.001) higher than the controls. A marked increase (p < 0.001) in vitamin A content of the thymus was also noted in animals treated with retinol alone, but the values were similar to those found in animals treated with the vitamin in combination with corticosterone.

Histological studies of the thymus revealed focal necrosis affecting the thymocytes of the cortical region in corticosterone-treated animals. These changes were not observed in animals treated with both corticosterone and retinol, or in the group given retinol alone or in the control groups.
Table 3.4  Effect of treatment with corticosterone and retinol for one week.

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>Corticosterone</th>
<th>Corticosterone + retinol</th>
<th>Retinol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma vitamin A (µg/100ml)</td>
<td>60 ± 2.8</td>
<td>47 ± 3.4**</td>
<td>66 ± 4.2</td>
<td>67 ± 2.7</td>
</tr>
<tr>
<td>Plasma vitamin E (µg/ml)</td>
<td>7.62 ± 0.66</td>
<td>5.47 ± 0.46*</td>
<td>7.9 ± 0.6</td>
<td>7.4 ± 0.6</td>
</tr>
<tr>
<td>Liver vitamin A concentration (µg/g)</td>
<td>162 ± 4.6</td>
<td>134 ± 8.2*</td>
<td>176 ± 7.3</td>
<td>186 ± 7.9*</td>
</tr>
<tr>
<td>Total liver vitamin A (mg/liver)</td>
<td>2.01 ± 0.11</td>
<td>1.57 ± 0.07**</td>
<td>2.17 ± 0.12</td>
<td>2.37 ± 0.13*</td>
</tr>
<tr>
<td>Thymus vitamin A (µg)</td>
<td>0.29 ± 0.038</td>
<td>0.09 ± 0.017***</td>
<td>0.60 ± 0.017***</td>
<td>0.70 ± 0.026***</td>
</tr>
<tr>
<td>Adrenal vitamin A (µg/pair)</td>
<td>0.51 ± 0.05</td>
<td>0.18 ± 0.03***</td>
<td>0.50 ± 0.05</td>
<td>0.96 ± 0.06***</td>
</tr>
</tbody>
</table>

Each value represents mean ± SEM for 6 rats.

* Significantly different from control. (* p < 0.05, ** p < 0.01, *** p < 0.001)
Pretreatment of normal rats with corticosterone (25mg/kg, twice daily) for four successive days resulted in a significant reduction in vitamin A status, as determined by decreases in the vitamin A levels in plasma, liver and adrenal glands. The corticosterone-mediated depression of the vitamin A content of the adrenals appeared parallel to the weight loss of the glands. The observed decrease in size of the adrenals may be due to a feedback mechanism resulting from a decreased necessity for corticosteroid production, in the presence of exogenous corticosteroids.

Of the tissues studied, thymus was the most sensitive to corticosterone treatment. Thus, steroid treatment for one day or more resulted in significant thymic involution and marked loss of vitamin A. It is noteworthy that loss of vitamin A from the plasma, adrenal glands and thymus has also been reported in mice subjected to physical stress by the application of a partial body cast to the thorax for 3 days (Rettura et al., 1976). In this study the greatest loss of vitamin A was from the thymus.

The corticosterone-mediated depression of vitamin A in the plasma, liver and adrenals was restored to normal levels by concomitant retinol administration. Steroid-treatment also decreased the plasma concentration of vitamin E. The ability of retinol to restore the depressed plasma vitamin E levels suggest a synergistic effect between the two vitamins as suggested by Bauernfeind et al. (1974).

However, upon administration of corticosterone in combination
with retinol, the most profound effect was seen in the thymus; for the vitamin A content rose to values about three times higher than the controls, without any significant increase in the size of the organ. It is also of interest that corticosterone-induced focal necrosis of the thymocytes in the cortical region was prevented by concomitant retinol administration. Thus, the thymus has the ability to accumulate vitamin A, even when involuted as a result of exogenous corticosteroid action. This seems to suggest a greatly increased requirement of the thymus for vitamin A.

An important function of the thymus is its involvement in immune reactions, particularly cell-mediated immunity. Seifter et al. (1973b) have shown that physical stress by partial body casting enhanced the development of tumours in mice inoculated with Moloney sarcoma virus. An associated decrease in weight of the thymus was observed, due mainly to loss of cortical cells. High doses of vitamin A decreased the incidence and severity of development of the viral sarcoma (Seifter et al., 1973c) and also of a transplantable melanoma (Felix et al., 1975) in mice. Furthermore, vitamin A administration reduced the incidence of viral tumours during immunosuppressive chemotherapy in transplantation and accelerated graft rejection (Rettura et al., 1975). The increased incidence of tumours may be due to reduced immunocompetence. In fact, Zisblatt and Lilly (1972) showed that animals with reduced immunocompetence had a markedly higher susceptibility to tumour induction. Therefore, it seems likely that the ability of vitamin A to restore immunocompetence under conditions associated with increased corticosteroid production is mediated by the thymus.

Recent work suggests that vitamin A stimulates both humoral and
cell-mediated immunity. Jurin and Tannock (1972) observed that intra-peritoneal injection of vitamin A accelerated the rejection of male skin grafts by isologous female recipient mice. An immune stimulating effect of vitamin A therapy has also been observed in children with Down's syndrome (Palmer, 1977).

It is possible that low vitamin A may favour tumour growth by compromising immune reactions which normally deal with a small number of transformed cells. Therefore, it is not surprising that several indices of immunological competence are impaired in patients with malignancies, particularly in the lung (Israel, 1973; Botton et al., 1975; Holmes, 1976). As described earlier (Chapter Two), serum vitamin A levels are decreased in patients with lung cancer. The present study suggests that elevated glucocorticoids may be a contributory factor in lowering vitamin A in lung cancer patients, particularly in oat cell carcinomas which may be associated with ectopic ACTH production. The failure to find this correlation in our patients could have been due to the fact that only five patients with oat cell carcinoma were studied.

There has been a recent claim that treatment with vitamin A (retinyl palmitate) or a synthetic analogue (13-cis retinoic acid) stimulated the immune response in lung cancer patients and reduced tumour progression (Micksche et al., 1977). However, the results were not very clear cut possibly due to the rather advanced nature of the disease. Therefore, it seems reasonable to suggest that the decreased vitamin A levels in lung cancer patients may in turn affect tumour growth by impairing cell-mediated immunity.
CHAPTER FOUR

VITAMIN A AND METASTATIC TESTICULAR TERATOMA IN MAN
4.1 INTRODUCTION

Abnormalities in vitamin A metabolism have been observed in patients with primary lung cancer (see Chapter Two), and may also occur in patients with other primary malignant tumours which give rise to metastases in the lung. Testicular teratoma is a highly malignant tumour which occurs in younger rather than older men and frequently spreads to the lung in most cases. It seemed to be of interest to study the vitamin A transport system and other related factors in such patients.

Moreover, aggressive chemotherapy used in the treatment of advanced testicular teratoma often has undesirable side effects (Editorial, Cancer Topics, 1979). In fact, toxicity may limit the amount of drugs which can be given to achieve significant remission. Furthermore, treatment with antineoplastic agents aggravates deficiencies of cancer patients who are already nutritionally compromised (Donaldson, 1977; Ohnuma and Holland, 1977; Donaldson and Lenon, 1979).

Two chemotherapeutic regimens currently employed in the treatment of metastatic testicular teratoma are:

1. Combination chemotherapy with vinblastine and bleomycin (Samuels et al., 1976).
2. Combination chemotherapy with cis-diamine dichloro platinum (II) - cis-DDP, vinblastine and bleomycin (Einhorn and Donohue, 1977).
The major toxic manifestations of vinblastine are, in decreasing order of frequency, leucopaenia, nausea, vomiting and anorexia, neurotoxicity and hair loss, and stomatitis, diarrhoea, constipation, lethargy and depression (Creasy, 1975). The organs more susceptible to the toxic side effects of bleomycin are the skin, lungs and mucous membranes (Bennet and Reich, 1979). Drug-induced pyrexia and nausea and vomiting are frequently observed (Blum et al., 1973) and the incidence of stomatitis is increased when it is combined with other antineoplastic agents, such as cis-DDP (Kaplan and Vogl, 1978). The major toxic effects of cis-DDP include gastro-intestinal, audiological, renal and haematological manifestations (Rozencwig et al., 1978). Thus, nutritional disturbances may also occur in patients receiving a combination of these drugs. Samuels and co-workers (1976) have observed a marked loss in body weight during chemotherapy with vinblastine and bleomycin.

No studies have so far been conducted to investigate the effects of these drugs on vitamin status. In the present study, the plasma level of vitamin A and other parameters which may affect its concentration in the plasma were studied in patients with metastatic testicular teratoma and the values compared with those of normal healthy subjects, to ascertain whether abnormalities in vitamin A metabolism also exist in these patients at the onset of treatment. The variation of plasma vitamin A and related factors was followed during chemotherapy and compared with that of thiamine, pyridoxine and ascorbic acid.
Nineteen patients with metastatic testicular teratoma admitted to the Royal Marsden Hospital, Sutton, Surrey were studied. Their ages ranged from 19 to 52 years with a mean age of $29.6 \pm 2.0$ years (Table 4.1). The tumours were classified according to their histology and staging (Peckham et al., 1979). The histological classification of the tumours is shown in Table 4.1, together with an abbreviated clinical description. The details of clinical staging, as described by tumour extent, site(s) and volume are as follows:

Stage I - Lymphogram negative, no evidence of metastases.

Stage II - Lymphogram positive, metastases confined to abdominal nodes, 3 sub-groups recognised:
- A - maximum diameter of metastases < 2cm;
- B - maximum diameter of metastases 2 - 5 cm;
- C - maximum diameter of metastases > 5cm.

Stage III - Involvement of supradiaphragmatic and infradiaphragmatic lymph-nodes.
No extralymphatic metastases.
Abdominal status: A, B, C as for Stage II.

Stage IV - Extralymphatic metastases.
Suffixes: O-lymphogram negative; A, B, C as for Stage II.
Lung status:
- $L_1 \leq 3$ metastases;
- $L_2$ multiple $\leq 2cm$ maximum diameter
- $L_3$ multiple $> 2cm$ diameter
Liver status:

H⁺ - liver involvement

The pretreatment plasma vitamin A levels and some related factors found in these patients were compared with those of 8 normal healthy male subjects (controls). The ages of the controls ranged from 19 to 34 years, with a mean value of 24.1 ± 2.2 years.

Of the 19 patients, it was possible to follow longitudinally, 7 patients being treated on the Samuels regimen and 7 patients being treated on the Einhorn regimen.

Samuels regimen: Each patient received at least four courses of therapy (with a maximum of six) at intervals of 4 - 5 weeks. The patients received vinblastine (15mg/m², intravenously on days 1 and 2) and bleomycin (30mg/day, administered as a continuous infusion over 24 hours on days 1 to 5). Overnight fasting blood samples were collected before starting treatment and on day 7 of each course.

Einhorn regimen: Each patient was given four courses of therapy, the duration of each course being 21 days. The patients received cis-DDP (20mg/m², intravenously, on days 1 to 5), vinblastine (0.2mg/kg, intravenously, on days 1 and 2) and bleomycin (30mg/day, intravenously, on days 2, 9 and 16). Blood samples were collected before treatment, on day 7 (before discharge from hospital) and on day 16.

The response to therapy in the patients studied longitudinally
Table 4.1 Clinical details of patients with testicular teratoma.

<table>
<thead>
<tr>
<th>Name</th>
<th>Age (years)</th>
<th>Previous treatment</th>
<th>Histological(^a) and clinical staging(^b) of the tumour</th>
</tr>
</thead>
<tbody>
<tr>
<td>G.T.W</td>
<td>34</td>
<td>Right orchidectomy</td>
<td>MTU, Stage IVC H(_+)</td>
</tr>
<tr>
<td>J.C.</td>
<td>42</td>
<td>Right orchidectomy</td>
<td>MTI, Stage IVO L(_3)</td>
</tr>
<tr>
<td>M.M.</td>
<td>25</td>
<td>Right orchidectomy</td>
<td>MTI, Stage IVC L(_2)</td>
</tr>
<tr>
<td>W.P.</td>
<td>22</td>
<td>Left orchidectomy, nephrectomy, ureterectomy and radiotherapy</td>
<td>MTU, Stage IIC</td>
</tr>
<tr>
<td>N.W.</td>
<td>28</td>
<td>Left orchidectomy</td>
<td>MTU, Stage IVO L(_2)</td>
</tr>
<tr>
<td>A.G.</td>
<td>34</td>
<td>Left orchidectomy</td>
<td>MTU, Stage IIIB</td>
</tr>
<tr>
<td>M.H.</td>
<td>33</td>
<td>Right orchidectomy</td>
<td>MTU, Stage IIC</td>
</tr>
<tr>
<td>G.S.</td>
<td>23</td>
<td>Right orchidectomy</td>
<td>MTU, Stage IVC H(_+)</td>
</tr>
<tr>
<td>G.S.</td>
<td>38</td>
<td>Right orchidectomy</td>
<td>MTI, Stage IVO L(_2)</td>
</tr>
<tr>
<td>D.M.</td>
<td>27</td>
<td>Right orchidectomy</td>
<td>MTU, Stage IVA L(_1)</td>
</tr>
<tr>
<td>P.D.</td>
<td>22</td>
<td>Left orchidectomy</td>
<td>MTI, Stage IVO L(_1)</td>
</tr>
<tr>
<td>A.S.</td>
<td>25</td>
<td>Right orchidectomy</td>
<td>MTI, Stage IIA</td>
</tr>
<tr>
<td>D.N.</td>
<td>52</td>
<td>Right orchidectomy</td>
<td>MTI, Stage IVO L(_3)</td>
</tr>
</tbody>
</table>

\(^a\) Histology:
MTU - Malignant teratoma undifferentiated.
MTI - Malignant teratoma intermediate.
MTT - Malignant teratoma trophoblastic.

\(^b\) For details See description of patients.
<table>
<thead>
<tr>
<th>Name</th>
<th>Age (years)</th>
<th>Previous treatment</th>
<th>Histological and clinical staging of the tumour</th>
</tr>
</thead>
<tbody>
<tr>
<td>J.T.</td>
<td>40</td>
<td>Left orchidectomy and radiotherapy</td>
<td>MTI, Stage IVO L₁</td>
</tr>
<tr>
<td>M.H.</td>
<td>23</td>
<td>Bilateral orchidectomy and radiotherapy</td>
<td>Right-MTI, Left-seminoma Stage IVO L₁</td>
</tr>
<tr>
<td>G.H.</td>
<td>32</td>
<td>Left orchidectomy</td>
<td>MTI, Stage IVC L₁</td>
</tr>
<tr>
<td>M.G.</td>
<td>19</td>
<td>Right orchidectomy</td>
<td>MTI, Stage IIIB</td>
</tr>
<tr>
<td>A.D.</td>
<td>23</td>
<td>Right orchidectomy and radiotherapy</td>
<td>MTU, Stage IVO L₂</td>
</tr>
<tr>
<td>A.S.O.</td>
<td>22</td>
<td>Left orchidectomy</td>
<td>MTU, Stage IVC L₃</td>
</tr>
</tbody>
</table>
is shown in Table 4.2.

Overnight fasting blood (15ml) was collected from each patient and control subject by venepuncture. An aliquot (1ml) was transferred to a 5ml heparinised tube, mixed and stored at -40°C. This was used to measure the stimulation of transketolase activity by thiamine pyrophosphate. Another portion (4ml) was transferred to a sequestrene tube, mixed gently and centrifuged. Aliquots (0.5ml) of the plasma were added to 2ml aliquots of 5% TCA. The solutions were mixed, centrifuged and the supernatant TCA extracts were used to determine plasma ascorbic acid. The remainder of the blood was transferred to a heparinised tube, mixed gently and centrifuged. The plasma was separated and aliquots were stored in sample tubes covered with foil at -40°C. The residue of red cells was washed with isotonic saline, centrifuged and the supernatant was discarded. The red cells were lysed by adding distilled water. The haemolysate was used to measure the stimulation of transaminase activity by pyridoxal 5-phosphate.

The haematocrit values of each blood sample was determined and used for the calculation of transketolase and transaminase activities.

Statistical significance was calculated using either the students t-test, or the paired t-test in longitudinal studies.
### Table 4.2.1. Response to therapy in patients treated on the Samuels regimen.

<table>
<thead>
<tr>
<th>Name</th>
<th>Response to therapy</th>
<th>Other information</th>
</tr>
</thead>
<tbody>
<tr>
<td>G.T.W.</td>
<td>Good response</td>
<td>Course of radiotherapy later. Recurrence 2 months after completing chemotherapy. Bulky lung metastases detected.</td>
</tr>
<tr>
<td>J.C.</td>
<td>Static after course I, progression later</td>
<td>Died later.</td>
</tr>
<tr>
<td>M.M.</td>
<td>Fairly good response, lung metastases regressed</td>
<td>Four more courses of chemotherapy and radiotherapy. Abdominal mass removed by surgery. Patient well.</td>
</tr>
<tr>
<td>N.W.</td>
<td>Good response, lung metastases regressed</td>
<td>Patient well.</td>
</tr>
<tr>
<td>W.P.</td>
<td>Good response, residual abdominal mass</td>
<td>Two more courses of chemotherapy and surgical removal of residual tumour. Patient well.</td>
</tr>
<tr>
<td>A.G.</td>
<td>Regression of tumour</td>
<td>VB treatment poorly tolerated. Developed paralytic ileus after course I and course II given 2 months later.</td>
</tr>
<tr>
<td>M.H.</td>
<td>Partial response</td>
<td>Radiotherapy later and surgical removal of abdominal mass, but patient unwell.</td>
</tr>
</tbody>
</table>
Table 4.2.2. Response to therapy in patients treated on the Einhorn regimen.

<table>
<thead>
<tr>
<th>Name</th>
<th>Response to therapy</th>
<th>Other information</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.S.</td>
<td>No evidence of active disease</td>
<td>Pulmonary function affected as a result of bleomycin treatment - developed 'bleomycin lung'</td>
</tr>
<tr>
<td>D.N.</td>
<td>Partial response</td>
<td>Asymptomatic.</td>
</tr>
<tr>
<td>J.T.</td>
<td>No change</td>
<td>Thoracotomy suggested.</td>
</tr>
<tr>
<td>M.H.</td>
<td>Good response</td>
<td>Thoracotomy suggested.</td>
</tr>
<tr>
<td>G.H.</td>
<td>Marked regression</td>
<td>Two further courses of chemotherapy.</td>
</tr>
<tr>
<td>M.G.</td>
<td>Regressing</td>
<td></td>
</tr>
<tr>
<td>A.S.O.</td>
<td>Dramatic clearing after course I</td>
<td>Died of septicaemia and cardiac arrest after course 3. Post mortem revealed persisting abnormality in the lung.</td>
</tr>
</tbody>
</table>

*Pneumonitis due to bleomycin accumulation.*
4.3 ANALYTICAL METHODS

The plasma concentrations of vitamins A and E, retinol-binding protein, prealbumin, proteins, copper and zinc were determined by the methods described in Chapter Two. Other methods used for the first time are described below.

4.3.1. Determination of plasma ascorbic acid.

Total ascorbic acid in the plasma was determined by the method of Denson and Bowers (1961). Total ascorbic acid, that is, ascorbic acid, dehydroascorbic acid and diketogulonic acid was determined by coupling with 2, 4 dinitrophenyl hydrazine, following oxidation of ascorbic acid. The resultant orange coloured compound was dissolved in sulphuric acid and the optical density was measured at 520nm using a Cecil spectrophotometer. The standard curve obtained is shown in Figure 4.1.

4.3.2. Determination of transketolase stimulation.

The extent of stimulation of transketolase enzyme activity in vitro by thiamine pyrophosphate (TPP), the co-enzyme form of thiamine, was used as an index of the thiamine status of the individual (Dreyfus, 1962). A stimulation of greater than 15% is considered to be an indicator of thiamine deficiency.

The transketolase activity in haemolyzed whole blood was determined by the micro-assay of Basu et al, (1974d). Transketolase catalyzes the following reaction:
Fig. 4.1. Standard curve for the determination of ascorbic acid
An aliquot (50μl) of whole blood was incubated with ribose 5-phosphate (3.2mM) in the presence and absence of thiamine pyrophosphate (2mM). The pH of the incubation mixture was maintained at 7.4. The reaction was stopped by adding 30% TCA, after incubation for 30 min at 37°C. The amount of sedoheptulose was measured by the colour produced in the presence of cysteine and sulphuric acid. The optical density was measured at 510nm and 540nm using a SP-500 spectrophotometer. The difference in optical density between 510 and 540nm gives a measure of the sedoheptulose concentration. The standard curve obtained is shown in Fig. 4.2. Transketolase activity (Tk) was expressed as μmoles of sedoheptulose formed/min/ml of haemolysate.

\[
\% \text{ Stimulation by TPP} = \frac{\text{TK}_{\text{with TPP}} - \text{TK}}{\text{TK}} \times 100
\]

4.3.3. Determination of transaminase stimulation.

It has been suggested that the in vitro stimulation of erythrocyte transaminase activity by pyridoxal 5-phosphate (B₆ PO₄) could be used as an index of vitamin B₆ status in man (Sauberlich and Raica, 1964; Cinnamon and Beaton, 1970). The activities of both glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) in the erythrocytes were measured in the presence and absence of
Fig 4.2. Standard curve for the determination of transketolase activity
pyridoxal 5-phosphate. GOT catalyzes the following reaction.

\[
\begin{align*}
\text{α-ketoglutarate} & \xrightarrow{\text{GOT}} \text{glutamate} \\
\text{aspartate} & \xleftarrow{\text{B}_6\text{P}_4} \text{oxaloacetate}
\end{align*}
\]

The GOT activity was measured by a modification (Cheney et al., 1965) of the method of Tonhazy et al. (1950). An aliquot (0.2ml) of the erythrocyte haemolysate was incubated with α-ketoglutarate and buffered aspartate (pH 7.4) at 37°C for 10 min. The reaction was stopped by adding TCA. The oxaloacetate produced in the reaction was converted to pyruvate by adding aniline citrate. Pyruvate was converted to its phenyl hydrazone by treatment with 2, 4, dinitrophenyl hydrazine. The phenyl hydrazone of pyruvate was extracted using water-saturated toluene. The colour produced by mixing an aliquot (1 ml) of the toluene extract with 2.5% ethanolic potassium hydroxide was measured at 525nm using a Cecil spectrophotometer.

In the determination of GPT which catalyses the reaction shown below, buffered alanine (pH 7.4) was used instead of buffered aspartate and 0.5ml of haemolysate was used.

\[
\begin{align*}
\text{α-ketoglutarate} & \xrightarrow{\text{GPT}} \text{glutamate} \\
\text{aniline} & \xleftarrow{\text{B}_6\text{P}_4} \text{pyruvate}
\end{align*}
\]

As pyruvate is produced by the reaction itself, the decarboxylation step using aniline citrate was omitted. The coloured
compound formed with 2, 4 dinitrophenyl hydrazine was measured as described for GOT. The standard curves for GOT and GPT are shown in Figures 4.3 and 4.4 respectively.

\[
\% \text{ Stimulation} = \frac{\text{Transaminase activity with } B_6\text{PO}_4 - \text{Transaminase activity}}{\text{Transaminase activity}} \times 100
\]

The percentage stimulation of GOT in the 8 control subjects ranged from 0 - 65% and the stimulation of GPT ranged from 0 - 30%.
Fig. 4.3. Standard curve for the determination of GOT

Fig. 4.4. Standard curve for the determination of GPT
4.4 RESULTS

4.4.1. Pre-treatment values.

The pre-treatment plasma concentrations of vitamin A, retinol-binding protein (RBP) and prealbumin in testicular teratoma patients were compared with those of a control group of healthy age-matched subjects. The plasma vitamin A levels in teratoma patients were significantly ($p < 0.01$) lower (mean value $= 50.2 \pm 3.1 \mu g/\text{ml}$) than that of the control group (mean value $= 71.0 \pm 7.3 \mu g/\text{100ml}$) (Fig. 4.5). Eight patients had vitamin A concentrations less than $45 \mu g/\text{100ml}$, but these low values did not correlate with the staging of the disease or the presence of lung metastases. Six of these patients had malignant teratomas of the undifferentiated histological type. About fifty percent of the patients had Vitamin A concentrations within the normal range. The plasma RBP level was also significantly ($p < 0.01$) lower, with a mean value of $4.61 \pm 0.26 \text{mg/100ml}$, as compared to the control group (mean value $= 5.97 \pm 0.31 \text{mg/100ml}$). Only 3 patients had RBP levels above the mean of the control group (Fig. 4.6). In addition, the plasma prealbumin concentrations of these patients were also significantly ($p < 0.01$) decreased (mean value $= 20 \pm 1.3 \text{mg/100ml}$) as compared to the control group (mean value $= 27 \pm 1.2 \text{mg/100ml}$). Moreover, about a third of the patients had very low concentrations of prealbumin (Fig. 4.7).

A highly significant correlation ($p < 0.001$, $\gamma = 0.73$) was observed between vitamin A and RBP concentrations in the plasma of teratoma patients, but not in the controls (Fig. 4.8). Furthermore,
Fig. 4.5. Plasma vitamin A levels in testicular teratoma patients (●) and in age-matched controls (○).

*** Significantly different from controls (p < 0.01)
Fig. 4.6. Plasma retinol-binding protein levels in teratoma patients (●) and in age-matched controls (○)

*** Significantly different from controls (p < 0.01)
Fig. 4.7. Plasma prealbumin levels in testicular teratoma patients (●) and in age-matched controls (●).

*** Significantly different from control (p < 0.01)
Fig. 4.8. Relationship between vitamin A and retinol-binding protein in the plasma of teratoma patients (●) and age-matched controls (○).

Patients: $y = 0.73$, $p < 0.001$, $y = 5.43 + 9.52x$

Controls: N.S.
the plasma RBP levels were significantly \((p < 0.02, \gamma = 0.52)\) correlated with the plasma prealbumin levels (Fig. 4.9). However, the correlation between vitamin A and prealbumin was not significant (Fig. 4.10).

The concentration of vitamin E was similar in both teratoma patients and controls (Table 4.3). The plasma concentration of zinc was comparable in both groups, while that of copper was slightly, but not significantly elevated (Table 4.3).

4.4.2. (a) Effect of chemotherapy (Samuels regimen).

The changes in body weights and plasma total protein and albumin during chemotherapy in teratoma patients is shown in Table 4.4. The body weights decreased during each course of chemotherapy and the effect was greatest during the second course. The plasma total protein and albumin concentrations were not significantly altered.

The plasma vitamin A concentration decreased after each course of chemotherapy and the change was significant in all courses except the first (Fig. 4.11). The concentration of vitamin A rose between the end of the first course of treatment and the beginning of the second. Furthermore, the plasma vitamin A levels showed a gradual stepwise elevation during successive courses of chemotherapy. The vitamin A concentration at the beginning of the fourth course was significantly higher than the pre-treatment value. It is also of interest that the plasma vitamin A concentration remained elevated in a patient (N.W) who responded to therapy, while it showed no improvement in a patient (J.C) whose tumour progressed (Fig. 4.12).
Fig. 4.9. Relationship between prealbumin and retinol-binding protein in the plasma of teratoma patients (●) and age-matched controls (○).

Patients: \( y = 0.52, \ p < 0.02 \quad y = 7.79 + 2.69x \)

Controls: N.S.
Fig. 4.10. Relationship between vitamin A and prealbumin in the plasma of teratoma patients (●) and age-matched controls (○). Patients $\gamma = 0.42$, N.S. Controls. N.S.
<table>
<thead>
<tr>
<th></th>
<th>Copper</th>
<th>Zinc</th>
</tr>
</thead>
<tbody>
<tr>
<td>(μmol/L)</td>
<td>(μmol/L)</td>
<td></td>
</tr>
<tr>
<td>Patients (19)</td>
<td>9.0 ± 0.48</td>
<td>12.0 ± 0.43</td>
</tr>
<tr>
<td>Controls (8)</td>
<td>8.9 ± 0.85</td>
<td>12.4 ± 0.38</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SEM for the number of subjects in parenthesis.
Table 4.4. Changes in body weights and plasma proteins during chemotherapy (Samuels regimen)

<table>
<thead>
<tr>
<th></th>
<th>Course 1</th>
<th></th>
<th>Course 2</th>
<th></th>
<th>Course 3</th>
<th></th>
<th>Course 4</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
<td>Post</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>76.7 ± 3.3</td>
<td>75.4 ± 3.7</td>
<td>71.9 ± 4.5</td>
<td>65.3 ± 2.2***</td>
<td>70.9 ± 4.2</td>
<td>68.2 ± 4.9</td>
<td>71.9 ± 8.7</td>
<td>69.2 ± 9.2*</td>
</tr>
<tr>
<td>Plasma total protein (g/L)</td>
<td>70 ± 2.4</td>
<td>66 ± 1.6</td>
<td>70 ± 3.4</td>
<td>69 ± 1.7</td>
<td>66 ± 2.7</td>
<td>68 ± 2.3</td>
<td>69 ± 2.9</td>
<td>67 ± 2.3</td>
</tr>
<tr>
<td>Plasma Albumin (g/L)</td>
<td>40 ± 1.4</td>
<td>37 ± 1.6</td>
<td>40 ± 1.9</td>
<td>38 ± 2.0</td>
<td>36 ± 1.5</td>
<td>38 ± 1.7</td>
<td>38 ± 1.8</td>
<td>39 ± 2.9</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SEM for 7 patients
* Significantly different from the respective pre-treatment value
(* p < 0.05, ** p < 0.02, *** p < 0.01)
Fig. 4.11. Variation of plasma vitamin A during chemotherapy (Samuels regimen)

Each point represents the mean ± SEM for 7 patients (last course 6 patients)

* Significant difference between pre and post treatment value of a course (* p < 0.05, ** p < 0.02)
+ Significant difference between pretreatment values of first and fourth courses (+ p < 0.05)
The concentration of RBP in the plasma also showed a similar stepwise variation (Fig. 4.13). A marked increase in the RBP level was observed at the start of the second course of chemotherapy. A decrease due to chemotherapy occurred after each subsequent course. The RBP concentration at the start of the fourth course was significantly ($p < 0.02$) higher than the pre-treatment value. The plasma prealbumin concentration also showed a similar variation with time, but it was not significantly decreased by chemotherapy (Fig. 4.14). However, a marked elevation of prealbumin levels occurred at the start of the second course and it persisted during subsequent courses of chemotherapy. It is also noteworthy, that the values at the start of the fourth course were significantly ($p < 0.01$) higher than pre-treatment values.

A highly significant decrease in plasma vitamin E was observed at the end of the first two courses of chemotherapy (Fig. 4.15). However, in contrast to vitamin A, a significant improvement in plasma vitamin E did not occur during successive courses of chemotherapy. The plasma vitamin C concentration did not change (Fig. 4.16) significantly during chemotherapy, although there was a slight gradual elevation during progressive treatment. The % TPP effect was markedly increased after each course of chemotherapy (Fig. 4.17). Similarly, the percentage stimulation of both GOT and GPT by pyridoxal 5-phosphate were significantly elevated after each course of treatment (Fig. 4.18).

The variation of zinc and copper concentrations in the plasma during chemotherapy is shown in Figure 4.19. The plasma zinc levels decreased during treatment, but the fall was significant only in the first and third courses of chemotherapy. The mean value at the end of
Fig. 4.13. Variation of plasma retinol-binding protein during chemotherapy (Samuels regimen)

Each point represents the mean ± SEM for 7 patients (last course 6 patients).

* Significant difference between pre and post-treatment values of a course (* p < 0.05, ** p < 0.02)
† Significant difference between pretreatment values of first and fourth courses (†† p < 0.02)
Fig. 4.14. Variation of plasma prealbumin during chemotherapy (Samuels regimen)

Each value represents mean ± SEM of 7 patients (last course 6 patients)

+ Significant difference between pretreatment values of the first and fourth courses (+++ p < 0.01)
Fig. 4.15. Variation of plasma vitamin E during chemotherapy (Samuels regimen).

Each point represents the mean ± SEM for 7 patients (last course 6 patients)

* Significant difference between pre and post-treatment values of a course (**p' < .01, ***p < .001)
Fig. 4.16. Variation of plasma vitamin C during chemotherapy (Samuels regimen).

Each point represents the mean ± SEM for 7 patients (last course 6 patients)
Fig. 4.17. Variation of % TPP effect in haemolysed whole blood during chemotherapy (Samuels regimen).

Each point represents the mean ± SEM for 7 patients (last course 6 patients).

* Significant difference between pre and post-treatment values of a course
  (* p < 0.05, ** p < 0.02, *** p < 0.01, **** p < 0.001)
Fig. 4.18. Variation of % stimulation of GOT (▲) and GPT (●) by pyridoxal 5-phosphate during chemotherapy (Samuels regimen)

Each point represents the mean ± SEM for 7 patients (last course 6 patients)
* Significant difference between pre and post-treatment values of a course (* p < 0.05, ** p < 0.02, *** p < 0.01)
the fourth course of treatment was almost identical to the pre-treatment value. In contrast, the plasma copper levels were elevated after treatment. The rise in the concentration of copper was highly significant after the third and fourth courses.

It is also noteworthy that the plasma zinc levels were positively correlated with both vitamin A (Fig. 4.20) and RBP (Fig. 4.21) in the plasma. Moreover, a stronger correlation ($\gamma = 0.49, p < 0.01$) was observed between zinc and RBP than with vitamin A.

4.4.2 (b) Effect of chemotherapy (Einhorn regimen).

The body weights of teratoma patients treated with the Einhorn regimen were markedly decreased as a result of chemotherapy (Table 4.5). As with the Samuels regimen, chemotherapy did not change the values for plasma total proteins and albumin (Table 4.5).

The variations of mean plasma vitamin A concentrations during chemotherapy are shown in Figure 4.22. The concentration of vitamin A in the plasma in these patients was within the normal range, and thus differed in this respect from the patients treated with Samuels regimen. The levels decreased during chemotherapy and the fall was greater by the sixteenth day of each course. However, the change by the sixteenth day was statistically significant only during the first and second courses. The magnitude of the fall did, in fact, decrease with successive treatments.

The plasma RBP levels did not fall until after the seventh day during the first two courses. At the sixteenth day, the values were significantly lower than the pre-treatment value of the course, for
Fig. 4.19. Variation of plasma zinc (○) and copper (▲) during chemotherapy (Samuels regimen)

Each point represents the mean ± SEM for 7 patients (last course 6 patients).

* Significant difference between pre and post-treatment values of a course

(* p < 0.05, *** p < 0.01, **** p < 0.001)
Fig. 4.20. Relationship between zinc and vitamin A in the plasma of teratoma patients given chemotherapy (Samuels regimen).

- Pre-treatment values
- Post-treatment values

\[ y = 0.38, p < 0.01 \]  
\[ y = 9.82 + 0.042x \]
Fig. 4.21. Relationship between zinc and retinol-binding protein in the plasma of teratoma patients given chemotherapy.

- Pre-treatment values
- Post-treatment values

\[ y = 0.49, p < 0.01 \]
\[ y = 8.62 + 0.63x \]
Table 4.5. Changes in body weights and plasma proteins during chemotherapy (Einhorn regimen)

<table>
<thead>
<tr>
<th></th>
<th>Course 1 (days)</th>
<th>Course 2 (days)</th>
<th>Course 3 (days)</th>
<th>Course 4 (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>7</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>Body weight</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>68.2±2.4</td>
<td>66.4±2.5</td>
<td>68.0±3.3</td>
<td>67.9±5.2</td>
</tr>
<tr>
<td>Plasma total</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>protein (g/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>69±2.0</td>
<td>70±3.0</td>
<td>70±2.0</td>
<td>73±1.2</td>
</tr>
<tr>
<td>Plasma Albumin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(g/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>38±1.0</td>
<td>37±2.0</td>
<td>35±2.0</td>
<td>36±2.0</td>
</tr>
</tbody>
</table>

Each value is the mean ± SEM for 7 patients.

* Significantly different from its pre-treatment value (** p < 0.02, *** p < 0.01, **** p < 0.001)
Fig. 4.22. Variation of plasma vitamin A during chemotherapy (Einhorn regimen)
Each point represents the mean ± SEM for 7 patients
* Significantly different from the respective pre-treatment value (*** p < 0.01)
courses 1 and 4 (Fig. 4.23). The concentration at the start of the fourth course was significantly (p < 0.02) higher than the pre-treatment value of the first course. The plasma prealbumin concentrations were only slightly decreased during chemotherapy and showed a gradual elevation during successive courses of chemotherapy (Fig. 4.24). As in the case of RBP, the plasma prealbumin level at the start of the fourth course of chemotherapy was significantly (p < 0.02) higher than the values at the start of chemotherapy.

The vitamin E concentration in the plasma fell significantly during the first three courses of chemotherapy with a rise between treatments (Fig. 4.25). The concentration of vitamin C in the plasma was not markedly altered by chemotherapy (Fig. 4.26). The percentage TPP effect increased during the first three courses of chemotherapy (Fig. 4.27) and showed no evidence of returning to the pre-treatment value during the last two courses. The percentage stimulation of erythrocyte transaminases (GOT and GPT) by pyridoxal 5-phosphate also increased during chemotherapy (Fig. 4.28). A greater increase in the stimulatory effect was observed with GPT than with GOT.

In contrast to Samuels regimen, the plasma zinc concentration of patients treated on the Einhorn regimen were not depressed, but slightly elevated (Fig. 4.29), and the increase was significant only during the fourth course. The plasma copper concentrations were also significantly elevated during the fourth course (Fig. 4.29). In contrast to the Samuels regimen, the plasma zinc concentrations were not correlated with either vitamin A or RBP.
Fig. 4.23. Variation of plasma retinol-binding protein during chemotherapy (Einhorn regimen).
Each point represents the mean ± SEM for 7 patients.
* Significantly different from the respective pre-treatment value (* p < 0.05)
** Significantly different from the pre-treatment value of the first course (++ p < 0.02)
Fig. 4.24. Variation of plasma prealbumin during chemotherapy (Einhorn regimen)
Each point represents the mean ± SEM for 7 patients
† Significantly different from the pre-treatment value of the first course (++ p < 0.02)
Fig. 4.25. Variation of plasma vitamin E during chemotherapy (Einhorn regimen)
Each point represents the mean ± SEM for 7 patients
* Significantly different from the respective pre-treatment value (* p < 0.05, *** p < 0.01)
Fig. 4.27. Variation of % TPP effect during chemotherapy (Einhorn regimen)
Each point represents the mean ± SEM for 7 patients
* Significantly different from the respective pre-treatment value (* p < 0.05, ** p < 0.01)
Fig. 4.28. Variation of the stimulatory effect of GOT (▲) and GPT (●) by pyridoxal 5-phosphate during chemotherapy (Einhorn regimen).

Each point represents the mean ± SEM for 7 patients.

* Significantly different from the respective pre-treatment value (* p < 0.05, ** p < 0.02)
Fig. 4.29. Variation of plasma zinc and copper during chemotherapy (Einhorn regimen)

Each point represents the mean ± SEM for 7 patients

* Significantly different from the respective pre-treatment value (*p < 0.05, ** p < 0.02, *** p < 0.01)
4.5. **DISCUSSION**

The pre-treatment concentrations of vitamin A and RBP were significantly lower in patients with metastatic testicular teratoma than in age-matched healthy male subjects. This observation is similar to that of patients with lung cancer (Chapter Two). Moreover, the observation of a correlation between vitamin A and RBP in teratoma patients, as in lung cancer patients, suggest that the low levels may be due to low levels of RBP, rather than a restricted dietary intake. There was considerable variation in the vitamin A concentration of teratoma patients, and the levels found in the five patients who did not have lung metastases were within the range of those that did have lung metastases. Also, there was no apparent correlation between the vitamin A levels and the staging of the tumour. However, it is of interest that six of the eight patients who had low vitamin A concentrations had malignant teratomas of the undifferentiated histological type. Low vitamin A levels were also observed in patients with undifferentiated lung cancer (see Chapter Two). This observation may be of great importance, in view of the role of vitamin A in controlling the differentiation of epithelial tissues. However, its significance is not clear at present.

In contrast to patients with primary lung cancer, patients with metastatic testicular teratoma had low prealbumin levels. Thus, the entire retinol-transport system was affected in the teratoma patients and the subnormal vitamin A concentrations may have resulted from impairment of this system. This is further supported by the observation of a correlation between RBP and prealbumin.
Low concentrations of vitamin A may occur in conditions associated with impaired fat absorption. However, the observation of similar concentrations of vitamin E in both teratoma patients and in age-matched control subjects implies that the low concentrations of vitamin A were not due to malabsorption of fat. Moreover, vitamin E is an important factor in the absorption and biological utilisation of vitamin A (Bauernfeind et al., 1974; Jagadeesan and Reddy, 1978). Thus, as in the lung cancer patients, the low plasma vitamin A levels were not a result of impaired absorption or utilisation due to lack of vitamin E.

It was suggested in Chapter Two that the low circulating levels of RBP could have been the result of a reduced availability of zinc. However, in patients with metastatic testicular teratoma, the plasma levels of both zinc and copper were similar to the controls. It thus seems possible that in these patients the low prealbumin and RBP may be a reflection of decreased hepatic synthesis due to liver damage by the tumour.

Treatment of these patients with either the Samuels or Einhorn regimens caused a marked fall in body weights. Samuels and co-workers (1976) have reported a median weight loss of 4.95 kg per course in patients with testicular tumours treated with their regimen, while adjunctive intravenous hyperalimentation resulted in a mean weight gain of 0.45 kg per course. In a recent review, Donaldson and Lenon (1979) have reported a mean weight loss of 2.9% of the body weight in 12 men with stage III non-seminomatous testicular carcinoma treated with the Einhorn regimen. In other studies (Copeland et al., 1975) the actual response to chemotherapy in patients with advanced cancer at various sites, such as the lung, colon and testis, was improved by intravenous
hyperalimentation. These workers also observed a positive correlation between the nutritional status and the response to therapy in patients with non-oat cell carcinoma of the lung. Although there is still some controversy as to whether nutritional supplementation of cancer patients feeds the patient or the tumour, evidence is accruing that in a number of circumstances, nutritional support is beneficial (Calmon, 1979) and there is no real evidence to the contrary in humans. Intravenous hyperalimentation though beneficial, introduces the patient to the risk of infection, particularly because of the impaired immunocompetence often found in cancer patients. Therefore, enteral nutrition may have a greater beneficial effect.

The patients with testicular teratoma showed other metabolic abnormalities during chemotherapy. Thus, the plasma vitamin A concentration fell on treatment with both chemotherapeutic regimens. However, there was a rise in the plasma vitamin A concentration to higher than pre-treatment values at the start of subsequent courses of chemotherapy in patients treated with the Samuels regimen, but not with the Einhorn regimen. This difference may be due to the fact that the patients given the Einhorn regimen had mean pre-treatment vitamin A levels within the normal range. The fall in vitamin A concentration was markedly reduced during the third and fourth courses of chemotherapy in patients treated with the Einhorn regimen. The elevation of vitamin A may seem to be of some prognostic significance, because the levels remained elevated in a patient who responded to chemotherapy with the Samuels regimen, whereas, the levels fell in a patient whose tumour was progressing. Such a comparison was not possible with the Einhorn regimen, as most of the patients responded
to treatment and no patient had tumour progression during treatment. The results of the two patients are in agreement with the finding by Soukop and Calmon (1978) of a poor response to chemotherapy which was associated with a low level of vitamin A.

The plasma RBP concentration in patients treated with the Samuels regimen followed a pattern similar to vitamin A, with a dramatic rise between the end of the first course and the start of the second course of chemotherapy. This rise was maintained at the start of subsequent courses with a fall during chemotherapy. In contrast, in patients treated with the Einhorn regimen, the plasma RBP levels rose initially and decreased to lower than pre-treatment values on the sixteenth day in the first two courses of therapy. The increase in RBP was observed after the period of treatment with cis-DDP. The kidney is the major organ involved in the catabolism of RBP, and elevated plasma RBP levels have been reported in patients with impaired renal function (Peterson, 1971). Cis-DDP is notably toxic to the kidney (Tally et al., 1978) and the transient rise in RBP may reflect its decreased catabolism as a result of disturbances of kidney function. The plasma RBP levels were elevated to higher than pre-treatment values of the first course at the start of subsequent courses of chemotherapy in both chemotherapeutic regimens. The plasma prealbumin concentration also followed a similar trend in both chemotherapeutic regimens rising to higher than pre-treatment values at the start of second and subsequent courses of chemotherapy.

Both RBP and prealbumin are synthesized in the liver (Smith and Goodman, 1971). It is possible that the presence of the tumour exerted a distant effect on the liver which was removed by chemotherapy. It
is noteworthy that only one patient whose vitamin profile was followed during chemotherapy had evidence of metastases in the liver. Thus, it seems unlikely that the change was due to dissemination of the tumour to the liver in other patients.

As early as 1957, Begg speculated that the presence of a tumour may cause metabolic disturbances in distant organs. Such metabolic abnormalities are now well documented (Theologides, 1972; Hall, 1974; Costa, 1977; Theologides, 1979). Cancers frequently produce peptides and other small molecules probably as a result of derepression of various genomes (Stonehill and Bendich, 1970; Hall, 1974; Islam, 1978). The hypothesis has been advanced that novel and common peptides and other small molecules frequently produced by the tumour may modify the activity of host enzymes through allosteric or other effects, activating and inactivating various enzymes in the tumour-free tissues of the host (Theologides, 1972; Theologides, 1974). These alterations in activities of host enzymes result in changes in various biochemical reactions and may throw the metabolism of the host into a chaotic state. However, conclusive evidence to support this hypothesis is still lacking.

When tumour growth is controlled by chemotherapy, the effects on some distant organs, such as the liver, in teratoma patients, may be removed and the normal synthesis of RBP and prealbumin could be restored. This would in turn increase the amount of vitamin A in circulation.

Chemotherapy also caused a fall in the concentrations of vitamin E and a decrease in status with respect to water-soluble
vitamins, thiamine and pyridoxine. These decreases may be due to reduced food intake and damage to the gastro-intestinal tract which are side effects common to all three antineoplastic agents (Ohnuma and Holland, 1977). With the Samuels regimen the values tended to return to pre-treatment values during the interval between courses. In contrast, in the Einhorn regimen, the percentage TPP effect showed no tendency to return to pre-treatment values in the last two courses. Thus, thiamine deficiency was aggravated by progressive treatment. In marked contrast to vitamin A, which is stored in the liver, the B vitamins, thiamine and pyridoxine are not stored in the body to any appreciable extent and are therefore dependent on normal dietary intake and absorption.

A fall in the concentration of plasma zinc was observed in patients treated with the Samuels regimen, but not the Einhorn regimen. Furthermore, the plasma zinc levels were positively correlated with both vitamin A and RBP in patients treated with the Samuels regimen. Thus, decreased zinc may be a contributory factor in affecting the synthesis or release of RBP during treatment with this regimen. The plasma zinc levels of patients treated with the Einhorn regimen, however, were not lowered by chemotherapy but in fact slightly elevated. This difference could be due to an alteration in the trace element balance by administration of large doses of a heavy metal complex, namely cis-DDP. Moreover, in contrast to Samuels regimen there was no correlation between zinc and RBP.
CHAPTER FIVE

EFFECT OF BLEOMYCIN AND VINBLASTINE ON NORMAL HEALTHY MALE RATS
5.1 INTRODUCTION

The antineoplastic agents, bleomycin and vinblastine, are widely used in the treatment of cancer and constitute the Samuels regimen for the treatment of metastatic testicular teratoma. The use of these drugs, however, has been frequently found to be associated with a number of side effects, including anorexia, nausea and vomiting and mucosal ulceration (Donaldson, 1977), which may in turn precipitate a number of nutritional problems.

Patients with lung cancer and metastatic testicular teratoma had low plasma levels of vitamin A and of its carrier protein, retinol-binding protein - RBP (see chapters 2 and 4). Moreover, the low RBP levels were associated with low plasma zinc levels in lung cancer patients and with low prealbumin levels in teratoma patients.

It was shown that the abnormalities in vitamin A metabolism in teratoma patients were exacerbated by treatment with cytotoxic drugs, such as vinblastine and bleomycin (see chapter 4). It is therefore of paramount importance to elucidate whether these drugs affect vitamin A metabolism in healthy animals. The present study was undertaken to investigate the effect of vinblastine and bleomycin, administered either singly, or in combination (in doses comparable to that of humans), on the plasma and tissues of normal healthy animals.
5.2 EXPERIMENTAL PROTOCOL

Normal healthy male Wistar-Albino rats weighing approximately 200g were used in all experiments. They were kept in individual cages and fed stock pellet food (Spiller) and water as described below.

5.2.1 Effect of bleomycin

Fifteen rats matched according to body weight were divided into three groups, each of five animals. Group 1 was given 0.1ml of 0.9% saline subcutaneously, and served as controls. Group 2 was given 0.1ml of bleomycin (Lundbeck Limited) in saline (3mg/kg body weight, twice daily, subcutaneously) and food and water ad libitum. Group 3 (pair-fed control) was given the same amount of food consumed by the bleomycin-treated animals and water ad libitum. All animals were treated for five successive days, their body weights and food and water intakes were measured daily. The animals were killed on the sixth day. Blood was collected by cardiac puncture into heparinised tubes and the plasma separated. The livers were removed and weighed, and the plasma and livers were stored at -40°C until analysed.

5.2.2 Effect of vinblastine

Fifteen rats, matched according to body weight were divided into three groups each of five animals. Group 1 was given 0.1ml of 0.9% saline intraperitoneally once daily, and food and water ad libitum. These animals served as controls. The animals in group 2 were given 0.1ml of vinblastine (Lilly Laboratories) in
0.9% saline (0.25mg/kg body weight, intraperitoneally, once daily) and food and water ad libitum. The animals in group 3 were pair-fed with the vinblastine group. All animals were weighed daily and the food and water intakes were measured. The animals were treated for two successive days and killed on the third day; they were bled and their livers removed as described earlier (5.2.1).

5.2.3 Effect of vinblastine in combination with bleomycin

In this study, vinblastine was administered in combination with bleomycin in a similar modality to that used in clinical practice.

Fifteen rats, matched according to body weight were divided into three groups, each of five animals. The animals in group 1 (vinblastine-bleomycin group) were treated with vinblastine in 0.9% saline (0.25mg/kg, body weight, intraperitoneally, once daily) on days 1 and 2, and bleomycin in saline (3mg/kg body weight, subcutaneously, twice daily) on days 1 to 5 inclusive. Group 2 was given food ad libitum and used as 'ad libitum controls', while group 3 was given the same amount of food consumed by the vinblastine-bleomycin group and used as pair-fed controls. All three groups were given water ad libitum. The animals were treated for four such courses with a two-day interval between courses. Their body weights and food intakes were measured daily. The animals were killed on the eighth day, after starting the fourth course of treatment; blood and livers were taken as described earlier (5.2.1).
5.2.4 Effect of vitamin A supplements on vinblastine treatment

Twenty four rats were divided into three groups, each of eight animals. Group 1 (vinblastine and vitamin A group) was given retinol (vitamin A) in corn oil (3000 I.U. per animal, intraperitoneally) and vinblastine in saline (0.25 mg/kg body weight, intraperitoneally) three hours later. Group 2 (vinblastine group) was given the same volume of corn oil intraperitoneally, and vinblastine (0.25 mg/kg body weight) three hours later. Group 3 was given the same amount of vehicle (corn oil and saline) and served as controls. All animals were given food and water ad libitum and the body weights and food and water intakes were measured daily. The animals were treated for two successive days and killed on the third day; their blood and livers were taken as described earlier (5.2.1).

5.2.5 Effect of vitamin A and zinc sulphate supplementation on vinblastine treatment

Thirty two rats were divided into four groups, each of eight animals. Group 1 (vinblastine, vitamin A and zinc sulphate group) was given retinol in corn oil (3000 I.U. per animal, intraperitoneally) and zinc sulphate in 0.9% saline (5 mg/kg body weight, intraperitoneally) and given vinblastine in 0.9% saline (0.25 mg/kg body weight, intraperitoneally) three hours later. Group 2 (vinblastine and vitamin A group) was given retinol in corn oil and vinblastine in saline three hours later. Group 3 (vinblastine group) was given the same volume of corn oil, and vinblastine three hours later. Group 4 was given the same volume of vehicle (corn oil and saline) and served as controls. All animals were given food and water ad libitum. Their body weights and food and water intakes were measured daily.
The animals were treated for two successive days and killed on the third day; their blood and livers were taken as described earlier (5.2.1).
The concentrations of vitamins A and E, zinc and proteins in the plasma were determined by the methods described in chapter two. The vitamin A in the liver was determined by the fluorometric method described in chapter three and the zinc content of the liver was determined by the method described below.

5.3. **Determination of zinc in the liver**

About 0.5g of liver homogenate was weighed accurately into each acid-washed conical flask and 5ml of concentrated nitric acid (A.R) and 1ml of perchloric acid (A.R) was added. The flasks were allowed to stand for 15 minutes and heated gently until the liver had completely dissolved. The temperature was gradually increased to 200°C and finally to 250°C when the solution became colourless. This temperature was maintained until the solution had completely evaporated to dryness. The resulting residue was redissolved in 10ml of 1% nitric acid with warming. Standard zinc solutions were prepared in 1% nitric acid. The zinc content was measured by atomic absorption spectroscopy as described for plasma zinc (chapter two). The standard curve obtained is shown in figure 5.1.
Fig. 5.1. Standard curve for the determination of tissue zinc.
5.4 RESULTS

5.4.1 Effect of bleomycin

Treatment of normal male rats with bleomycin (6mg/kg body weight/day) for five successive days resulted in a marked reduction in food intake (Table 5.1). The body weight gain of the bleomycin-treated animals was not only lower than that of the ad libitum controls, but also than that of the pair-fed controls. It appeared, therefore, that the reduced food intake as a consequence of bleomycin therapy accounts only in part for the restricted growth rate. Food restriction caused a decrease in liver weight relative to body weight in the pair-fed controls. Treatment with bleomycin did not have any effect on the absolute liver weight or liver weight relative to body weight when compared with ad libitum controls, but was slightly higher than in the pair-fed controls (Table 5.1).

The concentration of vitamin A in the plasma of the animals given bleomycin was lower than that of either the ad libitum ($p < 0.001$) or pair-fed ($p < 0.05$) controls (Table 5.2). The mean plasma vitamin A levels of the pair-fed controls were significantly lower than that of the ad libitum controls. The plasma vitamin E levels were significantly lower in both bleomycin-treated and pair-fed controls than in the ad libitum control group (Table 5.2). The vitamin A content of the liver showed a similar pattern to that of the plasma with the amount in the bleomycin-treated animals being significantly lower than that of either of the controls (Table 5.2).
Table 5.1. Effect of bleomycin (6mg/kg/day, subcutaneously for five successive days) on body weight, liver weight and food intake.

<table>
<thead>
<tr>
<th>Group</th>
<th>Ad libitum control</th>
<th>Bleomycin</th>
<th>Pair-fed control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight gain/animal (g)</td>
<td>32.2 ± 2.1</td>
<td>10.8 ± 2.9***</td>
<td>21.2 ± 3.1†</td>
</tr>
<tr>
<td>Total food intake/animal (g)</td>
<td>91.6 ± 2.2</td>
<td>66.2 ± 3.5***</td>
<td>66.2 ± 3.5</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>10.3 ± 0.4</td>
<td>9.2 ± 0.5</td>
<td>8.7 ± 0.2**</td>
</tr>
<tr>
<td>Liver weight/Body weight (%)</td>
<td>4.50 ± 0.15</td>
<td>4.38 ± 0.16*</td>
<td>3.95 ± 0.07***</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SEM for 5 rats.

† Significantly different from ad libitum control († p < 0.05, ††p < 0.01, †††p < 0.001).

* Significantly different from pair-fed control (* p < 0.05).
The plasma zinc concentration was not affected significantly by treatment with bleomycin, while the total amount of zinc was lower in the livers of both bleomycin-treated and pair-fed animals when compared with ad libitum controls (Table 5.2). The plasma total proteins and albumin were similar in the three groups (Table 5.2).

5.4.2 Effect of vinblastine

Giving vinblastine for two successive days caused a highly significant ($p < 0.001$) loss in body weight as compared to both pair-fed and ad libitum controls (Table 5.3). The gain in body weight in the pair-fed group was restricted to a mean value of 1.2g, but there was considerable difference between individual rats as is shown by the large standard error of the mean. Furthermore, the food intake of vinblastine-treated animals was significantly ($p < 0.001$) lower than that of ad libitum controls (Table 5.3). The liver of the vinblastine-treated rats weighed significantly less than that of either of the control groups both in absolute terms and relative to body weight (Table 5.3).

The plasma vitamin A concentration in the vinblastine-treated animals was significantly lower than that of either of the two control groups (Table 5.4). In contrast to the effect of bleomycin the plasma vitamin E levels were not significantly altered by vinblastine treatment. As with bleomycin, there was a decrease in the hepatic vitamin A content in parallel with the changes in plasma level (Table 5.4). The plasma zinc level, however, was slightly lower in both bleomycin-treated and pair-fed groups than in the ad libitum controls, while the total zinc content of the liver was
Table 5.2. Effect of bleomycin treatment for five successive days on vitamins A and E, zinc and plasma proteins.

<table>
<thead>
<tr>
<th>Group</th>
<th>Ad libitum control</th>
<th>Bleomycin</th>
<th>Pair-fed control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma vitamin A (µg/100ml)</td>
<td>62.3 ± 0.7</td>
<td>46.1 ± 0.9***</td>
<td>53.6 ± 2.1††</td>
</tr>
<tr>
<td>Plasma vitamin E (µg/ml)</td>
<td>6.46 ± 0.35</td>
<td>4.29 ± 0.21***</td>
<td>4.29 ± 0.12***</td>
</tr>
<tr>
<td>Total liver vitamin A (mg/liver)</td>
<td>1.40 ± 0.04</td>
<td>1.12 ± 0.04***</td>
<td>1.32 ± 0.05</td>
</tr>
<tr>
<td>Plasma zinc (µmol/L)</td>
<td>17.5 ± 1.0</td>
<td>15.5 ± 0.7</td>
<td>15.1 ± 1.1</td>
</tr>
<tr>
<td>Total liver zinc (mg/liver)</td>
<td>0.34 ± 0.01</td>
<td>0.27 ± 0.02***</td>
<td>0.28 ± 0.01***</td>
</tr>
<tr>
<td>Plasma total protein (g/L)</td>
<td>62.5 ± 1.0</td>
<td>60.3 ± 2.5</td>
<td>60.8 ± 2.0</td>
</tr>
<tr>
<td>Plasma albumin (g/L)</td>
<td>39.7 ± 1.2</td>
<td>38.7 ± 2.0</td>
<td>38.5 ± 1.5</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SEM for 5 rats.
+ Significantly different from ad libitum controls (**p < 0.01, ***p < 0.001).
* Significantly different from pair-fed controls (*p < 0.05, **p < 0.001)
Table 5.3. Effect of vinblastine (0.25mg/kg, intraperitoneally, for two successive days) on body weight, liver weight and food intake.

<table>
<thead>
<tr>
<th>Group</th>
<th>Ad libitum control</th>
<th>Vinblastine</th>
<th>Pair-fed control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight gain/animal (g)</td>
<td>10.4 ± 1.1</td>
<td>-12.8 ± 2.2***+++</td>
<td>1.2 ± 3.8†</td>
</tr>
<tr>
<td>Total food intake/animal (g)</td>
<td>43.8 ± 0.8</td>
<td>16.4 ± 4.1***+++</td>
<td>16.4 ± 4.1</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>9.7 ± 0.4</td>
<td>6.7 ± 0.5***+++</td>
<td>8.4 ± 0.5</td>
</tr>
<tr>
<td>Liver weight/Body weight (%)</td>
<td>5.2 ± 0.2</td>
<td>4.1 ± 0.2***+++</td>
<td>4.8 ± 0.1</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SEM for 5 rats.

† Significantly different from ad libitum controls († p < 0.05, ††p < 0.01, †††p < 0.001).

* Significantly different from pair-fed controls (* p < 0.05, ***p < 0.001)
significantly decreased only in the vinblastine-treated animals (Table 5.4).

The plasma total protein and albumin concentrations were significantly decreased by vinblastine treatment and this effect is independent of food intake (Table 5.4).

5.4.3 Effect of vinblastine in combination with bleomycin

The body and liver weights of rats treated with four courses of a combination of vinblastine and bleomycin are shown in Table 5.5. The body weight gain over the period of four courses of treatment was significantly lower in animals treated with vinblastine and bleomycin when compared with both pair-fed and ad libitum controls. Thus, during each course of treatment the restriction in body weight gain tended to be greater on days one to three (that is, during and immediately after treatment with vinblastine) (Fig. 5.2). Furthermore, the effect of each successive course on the overall weight gain appeared to increase. The gain in body weight was also restricted in the pair-fed animals, but to a lesser extent. The decrease in body weight during days one to three of each course was accompanied by a fall in food intake on those days (Fig. 5.3). The absolute liver weights were lower in both treated animals and pair-fed controls, than in the ad libitum controls, while the liver weight relative to body weight was lower only in pair-fed controls (Table 5.5).

The concentration of vitamin A in the plasma and the total amount in the liver were markedly reduced following treatment with vinblastine and bleomycin when compared with the values in the two
Table 5.4. Effect of vinblastine treatment for two days on vitamins A and E, zinc and plasma proteins.

<table>
<thead>
<tr>
<th>Group</th>
<th>Ad libitum control</th>
<th>Vinblastine</th>
<th>Pair-fed control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma vitamin A (µg/100ml)</td>
<td>61.1 ± 2.9</td>
<td>39.9 ± 2.5**++</td>
<td>56.3 ± 2.7</td>
</tr>
<tr>
<td>Plasma vitamin E (µg/100ml)</td>
<td>5.5 ± 0.36</td>
<td>4.81 ± 0.33</td>
<td>5.78 ± 0.34</td>
</tr>
<tr>
<td>Liver vitamin A (mg/liver)</td>
<td>1.04 ± 0.04</td>
<td>0.85 ± 0.04**+</td>
<td>1.00 ± 0.05</td>
</tr>
<tr>
<td>Plasma zinc (µmol/L)</td>
<td>16.9 ± 0.4</td>
<td>14.5 ± 0.9†</td>
<td>14.4 ± 0.9†</td>
</tr>
<tr>
<td>Total liver zinc (mg/liver)</td>
<td>0.28 ± 0.03</td>
<td>0.24 ± 0.01*†</td>
<td>0.26 ± 0.01</td>
</tr>
<tr>
<td>Plasma total protein (g/L)</td>
<td>61.3 ± 1.1</td>
<td>50.4 ± 3.9†</td>
<td>61.6 ± 2.3</td>
</tr>
<tr>
<td>Plasma albumin (g/L)</td>
<td>39.2 ± 1.5</td>
<td>28.2 ± 3.8†</td>
<td>38.7 ± 2.3</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SEM for 5 rats.

† Significantly different from ad. libitum controls († p < 0.05, ‡‡p < 0.01, ‡‡‡p < 0.001).

* Significantly different from pair-fed controls (* p < 0.05, ** p < 0.01).
Table 5.5. Effect of combination chemotherapy with four courses of vinblastine and bleomycin on body weight and liver weight.

<table>
<thead>
<tr>
<th>Group</th>
<th>Ad libitum control</th>
<th>Vinblastine and bleomycin</th>
<th>Pair-fed control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight (g)</td>
<td>176 ± 4</td>
<td>178 ± 3</td>
<td>177 ± 3</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>351 ± 8</td>
<td>253 ± ***†††</td>
<td>303 ± †</td>
</tr>
<tr>
<td>Total body weight gain/animal (g)</td>
<td>175 ± 9</td>
<td>74 ± ***†††</td>
<td>126 ± 4†</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>16.0 ± 0.6</td>
<td>12.0 ± 0.6††</td>
<td>11.9 ± 0.8††</td>
</tr>
<tr>
<td>Liver weight/Body weight (%)</td>
<td>4.6 ± 0.1</td>
<td>4.8 ± 0.2</td>
<td>3.9 ± 0.1†</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SEM for 5 rats.
† Significantly different from ad libitum controls (†p < 0.05, ††p < 0.01, †††p < 0.001).
* Significantly different from pair-fed controls (* p < 0.05, *** p < 0.001).
Fig. 5.2. Variation of body weight with time in vincristine + bleomycin-treated (●), pair-fed control (○) and ad libitum control (△) rats. Each point represents the mean body weight of 5 rats.

The animals were treated with vincristine (0.25mg/kg intraperitoneally on days 1 and 2) and with bleomycin (6mg/kg, subcutaneously on days 1, 2, 3, 4 and 5).
Fig. 5.3. Variation of food intake in vinblastine + bleomycin-treated (●) and ad libitum control rats (△). Each point represents the mean food intake of 5 rats.
control groups (Table 5.6). The pair-fed group had a mean hepatic vitamin A value which was intermediate between treated animals and ad libitum controls. Again, in contrast to vitamin A, the plasma vitamin E concentration was similar in the three groups (Table 5.6). The concentration of zinc in the plasma and the amount of zinc in the liver, was lower in the treated group than ad libitum controls, but not significantly lower than those that were pair-fed (Table 5.6).

The concentrations of plasma total proteins and albumin were significantly (p < 0.01) lower in the treated animals than in the pair-fed of ad libitum controls (Table 5.6).

5.4.4 Effect of vitamin A supplementation on vinblastine treatment

Table 5.7 shows that treatment with vinblastine for two successive days resulted in loss of body weight and that pretreatment of the animals with retinol (Vitamin A) counteracted the detrimental effect to some extent. Thus, the animals treated with vinblastine alone lost a mean weight of 6.6g, while the animals treated with vitamin A before administration of vinblastine had a mean body weight gain of 1.3g. As previously demonstrated, the loss of body weight in the vinblastine-treated animals was partly due to the anorectic effect of the drug. Pretreatment with vitamin A significantly reduced the anorectic effect, but did not restore the food intake to that of the controls (Table 5.7). Pretreatment with vitamin A did, however, prevent the significant loss of liver weight induced by the drug.

The administration of vitamin A with vinblastine partially
Table 5.6. Effect of combination chemotherapy with four courses of bleomycin and vinblastine on vitamins A and E, zinc and plasma proteins.

<table>
<thead>
<tr>
<th>Group</th>
<th>Ad libitum control</th>
<th>Vinblastine and bleomycin</th>
<th>Pair-fed control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma vitamin A (µg/100ml)</td>
<td>51.9 ± 1.5</td>
<td>34.5 ± 3.5 <strong>+++</strong></td>
<td>47.1 ± 2.7</td>
</tr>
<tr>
<td>Plasma vitamin E (µg/ml)</td>
<td>5.31 ± 0.35</td>
<td>5.72 ± 0.32</td>
<td>5.05 ± 0.47</td>
</tr>
<tr>
<td>Total liver vitamin A (mg/liver)</td>
<td>1.49 ± 0.02</td>
<td>1.08 ± 0.04 <strong>+++</strong></td>
<td>1.26 ± 0.06 ++</td>
</tr>
<tr>
<td>Plasma zinc (µmol/L)</td>
<td>14.9 ± 0.6</td>
<td>12.0 ± 0.8 †</td>
<td>12.9 ± 1.1</td>
</tr>
<tr>
<td>Total liver zinc (mg/liver)</td>
<td>0.45 ± 0.03</td>
<td>0.33 ± 0.02 ++</td>
<td>0.34 ± 0.08 †</td>
</tr>
<tr>
<td>Plasma total protein (g/L)</td>
<td>69.0 ± 3.5</td>
<td>58.6 ± 2.0 **+</td>
<td>63.4 ± 0.9</td>
</tr>
<tr>
<td>Plasma albumin (g/L)</td>
<td>46.9 ± 2.7</td>
<td>36.1 ± 2.5 **+</td>
<td>43.2 ± 1.2</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SEM for 5 rats.

† Significantly different from ad libitum controls († p < 0.05, ††p < 0.01, †††p < 0.001).
* Significantly different from pair-fed controls (*** p < 0.01, **** p < 0.001).
Table 5.7. Effects of vinblastine either alone, or in combination with vitamin A on body weight, liver weight and food intake.

<table>
<thead>
<tr>
<th>Group</th>
<th>Ad libitum control</th>
<th>Vinblastine and vitamin A</th>
<th>Vinblastine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight gain/animal (g)</td>
<td>15.4 ± 1.5</td>
<td>1.3 ± 2.7†††</td>
<td>-6.6 ± 2.6†††</td>
</tr>
<tr>
<td>Total food intake/animal (g)</td>
<td>40.5 ± 2.0</td>
<td>26.1 ± 2.9††</td>
<td>18.2 ± 1.7†††</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>9.8 ± 0.4</td>
<td>9.1 ± 0.4*</td>
<td>7.9 ± 0.3</td>
</tr>
<tr>
<td>Liver weight/BODY weight (%)</td>
<td>4.5 ± 0.10</td>
<td>4.4 ± 0.11**</td>
<td>3.9 ± 0.06</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SEM for 8 rats.

† Significantly different from ad libitum controls (†† p < 0.01, †††p < 0.001)

* Significantly different from vinblastine-treated rats (* p < 0.05, ** p < 0.01).
prevented the fall in plasma vitamin A concentration caused by the drug when given alone (Table 5.8). Giving vitamin A not only prevented the fall in hepatic vitamin A produced by the drug, but raised the level to higher than that found in the controls (Table 5.8). The plasma vitamin E and zinc concentrations and the zinc content of the liver were similar in the three groups (Table 5.8).

The decrease in the plasma total protein and albumin concentration was prevented by treatment of the animals with vitamin A before vinblastine administration (Table 5.8).

5.4.5 Effect of vitamin A and zinc sulphate supplementation on vinblastine treatment

Giving zinc sulphate in addition to vitamin A before administration of vinblastine did not enhance the beneficial effect of vitamin A in reducing the loss in body and liver weights and increasing the food intake (Table 5.9).

Furthermore, there was no improvement in the plasma and hepatic vitamin A status of rats pretreated with zinc sulphate and vitamin A over those caused by pretreatment with vitamin A alone (Table 5.10). However, the plasma zinc level was significantly (p < 0.01) raised in zinc sulphate and vitamin A pretreated animals compared with the other three groups (Table 5.10). The hepatic zinc content was only slightly increased in the former group. The plasma protein levels of the vitamin A and zinc sulphate pretreated rats were similar to those pretreated with vitamin A alone (Table 5.10).
Table 5.8. Effects of vinblastine either alone, or in combination with vitamin A on vitamins A and E, zinc and plasma proteins.

<table>
<thead>
<tr>
<th>Group</th>
<th>Ad libitum control</th>
<th>Vinblastine and vitamin A</th>
<th>Vinblastine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma vitamin A (μg/100ml)</td>
<td>50.4 ± 1.0</td>
<td>42.5 ± 2.1††</td>
<td>36.6 ± 1.4†††</td>
</tr>
<tr>
<td>Plasma vitamin E (μg/ml)</td>
<td>6.50 ± 0.50</td>
<td>6.59 ± 1.27</td>
<td>4.83 ± 0.64</td>
</tr>
<tr>
<td>Total liver vitamin A (mg/liver)</td>
<td>1.27 ± 0.03</td>
<td>1.40 ± 0.03†††</td>
<td>1.03 ± 0.05††</td>
</tr>
<tr>
<td>Plasma zinc (μmol/L)</td>
<td>17.8 ± 0.3</td>
<td>15.6 ± 0.5</td>
<td>15.5 ± 0.5</td>
</tr>
<tr>
<td>Total liver zinc (mg/liver)</td>
<td>0.27 ± 0.01</td>
<td>0.27 ± 0.01</td>
<td>0.25 ± 0.01</td>
</tr>
<tr>
<td>Plasma total protein (g/L)</td>
<td>60.4 ± 0.5</td>
<td>59.2 ± 0.7</td>
<td>53.4 ± 0.7†</td>
</tr>
<tr>
<td>Plasma albumin (g/L)</td>
<td>40.0 ± 0.3</td>
<td>38.7 ± 0.7†</td>
<td>30.3 ± 0.5††</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SEM for 8 rats.
† Significantly different from ad libitum control († p < 0.05, ††p < 0.01, †††p < 0.001)
* Significantly different from vinblastine-treated rats (* p < 0.05, ** p < 0.01, *** p < 0.001).
Table 5.9. Effect of vinblastine either alone, or in combination with vitamin A, or vitamin A and zinc sulphate on body weight, liver weight and food intake.

<table>
<thead>
<tr>
<th>Group</th>
<th>Ad libitum control</th>
<th>Vinblastine + vitamin A</th>
<th>Vinblastine + vitamin A + zinc sulphate</th>
<th>Vinblastine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight gain/animal (g)</td>
<td>12.5 ± 1.8</td>
<td>-2.75 ± 5.0**+++</td>
<td>-3.25 ± 5.9**+++</td>
<td>-14.2 ± 8.0+++</td>
</tr>
<tr>
<td>Total food intake/animal (g)</td>
<td>43 ± 2.5</td>
<td>23.0 ± 2.1**+++</td>
<td>23.4 ± 3.2**+++</td>
<td>15.5 ± 1.9+++</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>9.9 ± 0.2</td>
<td>8.3 ± 0.3†</td>
<td>8.1 ± 0.3†</td>
<td>7.4 ± 0.26+++</td>
</tr>
<tr>
<td>Liver weight/Body weight (%)</td>
<td>4.77 ± 0.21</td>
<td>4.25 ± 0.28**+++</td>
<td>4.19 ± 0.35**+++</td>
<td>3.9 ± 0.3+++</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SEM for 8 rats.

† Significantly different from ad libitum controls († p < 0.05, ++p < 0.01, +++p < 0.001).

* Significantly different from vinblastine-treated rats (* p < 0.05, ** p < 0.01).
<table>
<thead>
<tr>
<th>Group</th>
<th>Ad libitum control</th>
<th>Vinblastine + vitamin A</th>
<th>Vinblastine + vitamin A + zinc sulphate</th>
<th>Vinblastine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma vitamin A (µg/100ml)</td>
<td>53.7 ± 1.3</td>
<td>44.9 ± 1.1^†</td>
<td>45.9 ± 1.8^†</td>
<td>31.2 ± 1.6^+++</td>
</tr>
<tr>
<td>Plasma vitamin E (µg/ml)</td>
<td>6.92 ± 0.44</td>
<td>4.98 ± 0.44^†</td>
<td>6.10 ± 0.52^†</td>
<td>4.40 ± 0.51^†</td>
</tr>
<tr>
<td>Total liver vitamin A (mg/liver)</td>
<td>1.39 ± 0.03</td>
<td>1.56 ± 0.03^***‡‡</td>
<td>1.53 ± 0.03^***‡‡</td>
<td>1.00 ± 0.05^+++</td>
</tr>
<tr>
<td>Plasma zinc (µmol/L)</td>
<td>15.6 ± 0.55</td>
<td>14.8 ± 0.46</td>
<td>17.0 ± 0.35^†</td>
<td>14.9 ± 0.44</td>
</tr>
<tr>
<td>Total liver zinc (mg/liver)</td>
<td>0.27 ± 0.01</td>
<td>0.26 ± 0.01^*</td>
<td>0.27 ± 0.00^*</td>
<td>0.23 ± 0.01^‡‡</td>
</tr>
<tr>
<td>Plasma total protein (g/L)</td>
<td>59.3 ± 1.5</td>
<td>58.6 ± 2.0^*</td>
<td>58.0 ± 2.1^*</td>
<td>50.4 ± 1.5^†</td>
</tr>
<tr>
<td>Plasma albumin (g/L)</td>
<td>37.2 ± 1.7</td>
<td>34.7 ± 2.0^*</td>
<td>35.0 ± 2.1^*</td>
<td>26.2 ± 3.2^‡‡</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SEM for 8 rats.

† Significantly different from ad libitum control; (^p < 0.05, ‡‡p < 0.01, ‡‡‡p < 0.001)

* Significantly different from vinblastine-treated rats (*p < 0.05, **p < 0.01, ***p < 0.001).
In agreement with the observations on cancer patients (Ohnuma and Holland, 1977), the administration of both bleomycin and vinblastine to normal healthy male rats caused anorexia, with a consequent decrease in body weight gain. In fact, vinblastine though given for only two days caused a substantial decrease in body weight with a greater decrease in food consumption. Moreover, when animals were treated with a combination of the two drugs in a regimen comparable to that administered to human patients with metastatic testicular teratoma, a greater decrease in food intake and body weight gain was observed during and after vinblastine administration. The body weight gained by the rats decreased considerably during the third and fourth course so that the curve for the body weight diverged considerably from that of the pair-fed controls. Thus the effect of the drugs on body weight gain was not entirely accounted for by the anorectic effect of these drugs. It was clear that there was an additional toxic effect on the body tissues.

The only organ that was weighed in these studies was the liver. The decrease in liver weight caused by bleomycin was entirely accounted for by the decreased food intake although there was a suggestion that the liver was affected rather less than the remainder of the body. Vinblastine, on the other hand, caused a fall in liver weight which was greater than expected for the reduced food intake. The effect on the liver was relatively rather greater than that on the remainder of the body. Hence there was a small fall in the percentage contribution of the liver to the body weight.
bleomycin and vinblastine were given together the effect of vinblastine on the liver appeared to be counterbalanced by bleomycin.

Both bleomycin and vinblastine, when given independently or combined in courses caused a fall in plasma and liver vitamin A. Thus both drugs caused a mobilisation of vitamin A from the liver to help to maintain the plasma concentration. In view of the fact that the vitamin A concentration fell in the plasma as well as in the liver, it seems unlikely that the decrease in plasma vitamin A was simply due to impaired mobilisation. It is also possible that the trauma caused by treatment with these cytotoxic agents increased the requirement for vitamin A. In fact, Levenson et al. (1972) have shown that the requirement for the vitamin is increased by wounding and other forms of trauma.

The plasma total protein and albumin concentrations were not affected by bleomycin treatment, while a significant decrease occurred due to vinblastine administered either alone, or in combination with bleomycin. It is to be noted that the effect of vinblastine on the general body tissues was considerable.

Thus, the toxic manifestations of these two antineoplastic agents occur not only in patients with neoplastic disease, but also in healthy experimental animals.

In a study to determine whether some of these effects could be alleviated by vitamin A, the animals were treated with retinol (vitamin A) three hours prior to administration of vinblastine. The loss in body weight was drastically curtailed in the vitamin A pretreated animals and, in fact, a slight body weight gain was
observed. Moreover, the food intake was also increased in animals pretreated with vitamin A. Furthermore, the decrease in liver weight was prevented by treatment of the animals with vitamin A.

It thus appears that giving vitamin A reduces some of the detrimental effects of vinblastine on body weight, liver weight and food intake. Moreover, the fall in plasma proteins on treatment with vinblastine was also prevented by pretreatment with vitamin A. Pretreatment of rats with zinc sulphate in addition to vitamin A did not enhance the beneficial effects of the latter.

Several studies have shown that vitamin A enhances the action of some antitumour agents such as cyclophosphamide (Anton and Brandes, 1968) and 1,3 bis (2-chloroethyl) 1-nitroso urea - BCNU (Cohen and Carbone, 1972) in mice. Furthermore, vitamin A accelerates the regression of a skin tumour, keratoacanthoma, by 5-fluorouracil in rabbits (Prutkin, 1973).

The present study indicates that vitamin A alleviates some of the adverse effects of the antineoplastic agent, vinblastine. And, it is tempting to suggest that vitamin A may have the same beneficial effects in patients treated with vinblastine, either singly or in combination.
CHAPTER SIX

GENERAL DISCUSSION
It is now well-known that vitamin A plays an important role in growth, reproduction, visual function and the differentiation of epithelial tissues. It is also involved in the maintenance of mesenchymal structures, such as cartilage and bone (Moore, 1967). There have been a number of reports based largely on the results of studies in experimental animals which suggest that vitamin A deficiency is related to the genesis of cancer of epithelial tissues, such as those of the respiratory and genitourinary tracts (Sporn et al., 1976). Biochemical findings in a number of studies also suggest an association between low plasma vitamin A levels and cancer of some epithelial tissues in man. For example, vitamin A deficiency has been reported in patients with cancer of the stomach (Abels et al., 1941) and cervix (Wynder, 1969). However, the low plasma vitamin A levels observed in patients with cancer of the stomach may be largely due to a prolonged poor dietary intake and absorption in the presence of the slow growing tumour. Recently, Ibrahim et al. (1977) observed low plasma levels of vitamin A in 124 males and 79 females with squamous cell carcinoma of the oral cavity and oropharynx when compared to controls matched for age and sex.

In the present study, subnormal circulating vitamin A levels were observed in both groups of patients studied, namely patients with lung cancer and those with advanced testicular teratoma, many of whom had metastases in the lung. The lung cancer patients had serum vitamin A concentrations lower than patients having non-malignant lung diseases, or other non-malignant diseases. The plasma vitamin A levels of patients with metastatic testicular teratoma were lower than those of healthy age-matched male subjects. The
studies on lung cancer patients confirm the earlier work of Basu and co-workers (1976), who found the plasma vitamin A levels of lung cancer patients to be significantly lower than those of patients having non-malignant lung diseases or age-matched healthy subjects. The low circulating vitamin A levels may have been caused in a number of ways, some of which have been examined in this study.

Restricted dietary intake

There is epidemiological evidence pointing to an association between low dietary intake of vitamin A and the incidence of lung cancer. Bjelke (1975) in a retrospective study of 8278 residents in Norway found that low dietary vitamin A intake was negatively associated with lung cancer at all levels of smoking. However, as pointed out earlier (p.20) the results of this study must be viewed with caution as it did not take into consideration the ingestion of synthetic vitamin preparations or vitamin A-rich animal foods such as liver. In another study, Mettlin and co-workers (1979) observed that high dietary intake of vitamin A reduces the relative risk of development of lung cancer, particularly in heavy smokers. These studies are suggestive of a role for the vitamin in the aetiology of lung cancer. There are obvious difficulties in establishing such a relationship and what appears to be necessary is a long term combined epidemiological and biochemical study of a population 'at risk' extending over several decades.

Impaired absorption

Vitamin A or its precursor is absorbed into the intestinal
mucosal cells along with the products of digestion of dietary fat and re-esterified within the intestinal mucosa. The retinyl esters so formed are circulated as constituents of chylomicrons. Thus, low plasma levels of vitamin A might result from the malabsorption of fat. However, the finding of normal levels of vitamin E, another fat soluble vitamin in both groups of patients tends to exclude this possibility.

**Protein deficiency**

Depressed plasma vitamin A levels have been observed in conditions associated with protein deficiency. For example, in a group of Egyptian children with protein-energy malnutrition, low serum concentrations of vitamin A, RBP and prealbumin were observed along with low concentrations of albumin and total protein (Smith et al., 1973b). Supplementation with calories and protein without concomitant vitamin A therapy was found to cause a gradual elevation of vitamin A, RBP and prealbumin to normal levels. Further evidence for the requirement of dietary protein as a source of amino acids from which RBP can be synthesized for normal retinol transport to be established, has been reported in studies on a group of malnourished children in India (Venkataswamy et al., 1977). Low plasma protein levels may occur in cancer patients either due to decreased synthesis (Steinfeld, 1960; Mariani et al., 1976), or to loss from the intestine (Waldmann et al., 1977). However, the plasma total protein and albumin levels were within the normal range in the two groups of patients studied. These observations suggest that the low vitamin A levels observed in lung cancer patients and those with metastatic testicular teratoma were not a manifestation of generalised nutritional deficiency, but rather a specific effect on vitamin A metabolism.
Impaired mobilisation

In contrast to plasma proteins, the circulating RBP levels were significantly lower in patients with lung cancer, as well as in those with metastatic testicular teratoma. Moreover, the plasma RBP levels showed a strong positive correlation with plasma vitamin A levels in both groups of patients. Thus, it is possible that the low vitamin A levels arose as a result of low concentrations of RBP. Or alternatively, low vitamin A levels might prevent the secretion of RBP from the liver, as has been noted in vitamin A-deficient experimental animals (Muto et al., 1972). The block in RBP secretion seen after vitamin A depletion is highly specific for RBP. Thus, neither vitamin A depletion and deficiency, nor retinol repletion of deficient rats significantly altered plasma levels of prealbumin (Navab et al., 1977).

The patients with metastatic testicular teratoma differed from the lung cancer patients however, in having low plasma prealbumin levels in addition to low RBP levels. Thus, the low concentrations of plasma vitamin A and RBP in the teratoma patients could not have been due to dietary vitamin A deficiency, but may reflect impaired mobilisation as a result of decreased hepatic synthesis of RBP and prealbumin.

Very little is known about the mechanism of secretion of RBP from the liver. Figure 6.1 summarises the information available on the metabolism of vitamin A and RBP in the liver cell.
RBP synthesized in the microsomal fraction of the liver associates with retinol released from the hydrolysis of retinyl esters and is secreted into the circulation via the Golgi apparatus (Glover et al., 1974; Smith and Goodman, 1979). The secretion of RBP and prealbumin appear to be two independent processes with the formation of RBP-prealbumin complex occurring in the plasma (Smith and Goodman, 1979). These workers suggest that molecular signals from peripheral tissues may depress or stimulate the synthesis and secretion of RBP, and hence vitamin A, from the liver. However, the nature of these signals has not yet been elucidated.

It is of interest that, in the patients with testicular teratoma, the release of vitamin A, RBP and prealbumin from the liver seemed
to be impaired, although these patients (except for two) had no
evidence of spread of the malignant disease to the liver. Thus,
it seems possible that the synthesis and possibly the secretion
of RBP may be impaired by damage to the liver, by a distant effect
in the presence of the tumour elsewhere. Theologides (1979)
speculated that tumours synthesize novel and common polypeptides
or other molecules which are released into the circulation and cause
profound metabolic alterations in the function of tumour-free organs
of the host. Rapp (1973) and Calman and McAllister (1975) explained
the lower coenzyme A levels in the livers of tumour-bearing animals
when compared to their normal counterparts by suggesting that these
differences may be due to metabolic abnormalities occurring in the
non-involved organs of the tumour-bearing host.

In contrast to the situation in patients with testicular teratoma,
lung cancer patients had serum prealbumin levels comparable to those
of controls. However, lung cancer patients had subnormal serum zinc
levels, which were related to low vitamin A and RBP levels. Other
workers have also reported low circulating zinc levels in patients
with lung cancer (Davies et al., 1968; Davies, 1972; Andrews, 1979).
Thus, the low vitamin A levels seem to be in some way related to
the deficiency of zinc.

A relationship between zinc and vitamin A metabolism has been
demonstrated in studies on experimental animals (Smith et al., 1974;
Duncan and Hurley, 1978; Ette et al., 1979). An association
between zinc deficiency and vitamin A metabolism has been reported
in patients with alcoholic cirrhosis (Morrison et al., 1978), in
healthy adolescents (Michaelsson et al., 1976), and in male subjects
with severe acne (Michaelsson et al., 1977). A study on zinc-
deficient rats suggest that the low plasma vitamin A levels seen in zinc deficiency can be attributed to an impaired ability of the zinc-deficient rat to mobilise vitamin A from the liver as the RBP complex (Smith et al., 1974). These workers suggest that zinc-deficiency interferes with the synthesis of RBP by the liver.

It is also possible that zinc exerts its effect on vitamin A metabolism at other sites. Smith and Goodman (1979) speculate on the possibility that microsomal RBP may represent an intra-hepatic precursor - pro RBP, which is converted to RBP during the process of secretion. In view of the fact that zinc is a co-factor for proteolytic enzymes such as carboxypeptidases in man and other animals (Underwood, 1977) it seems reasonable to suggest that the conversion of such a 'pro-RBP' into an active RBP molecule might be a potential zinc-dependent regulatory step of RBP metabolism and hence of the mobilisation of vitamin A. Studies on the mechanism of synthesis and secretion of RBP and the role of zinc in RBP metabolism might provide an insight into the causation of altered vitamin A metabolism in some cancer patients.

**Corticosteroids**

In addition, a study was conducted to explore the effects of exogenous corticosteroids on vitamin A status in rats. This study revealed that high doses of corticosteroids, markedly depress vitamin A concentrations in the plasma, liver, adrenal glands and thymus. However, the effect of corticosteroids was greatest on the thymus, in which marked thymic shrinkage was accompanied by a rapid depletion of vitamin A. A similar effect was also seen in animals
subjected to stress (Seifert et al., 1976). Retinol (vitamin A) treatment concomitantly with corticosteroids had a profound effect on the thymus, for the vitamin A rose to values three times higher than controls. The ability of the thymus to accumulate vitamin A in the presence of corticosteroids suggests that there was an increased requirement for vitamin A.

The thymus plays a key role in cell-mediated immunity. The increased requirement for vitamin A in the thymus is suggestive of an antagonistic effect of vitamin A on the immunosuppressive effects of corticosteroids. Seifert et al. (1978) have shown that vitamin A inhibits the growth of some immunogenic tumour systems, and that this effect is related to the prevention of thymic involution and/or proliferation that accompanies tumour growth. Thus, it is possible that low vitamin A might cause impaired immunoresponsiveness and decrease the ability of the host to deal with a small number of transformed cells (Rettura et al., 1976). It is of interest that several indices of immunocompetence are depressed in patients with cancer, and particularly in those with lung cancer (Al-Saaraf et al., 1972; Wells et al., 1973; Brugarolas and Takita, 1973). In fact, stimulation of the immune response during therapy with retinyl palmitate or 13-cis retinoic acid has been reported in a study involving 9 male patients with metastatic, unresectable, squamous cell carcinoma, who had not received previous treatment (Micksche et al., 1977). The authors suggested that there was some response to vitamin A therapy, but the results appear to be inconclusive.

Chemotherapy

It was considered that the treatment, such as chemotherapy,
given to control malignant disease might well aggravate the abnormalities in vitamin A metabolism, since antineoplastic agents are not selective with respect to malignant cells, but also damage cells with a high turnover rate such as those of the gastro-intestinal tract. The combination chemotherapeutic regimens used in the treatment of metastatic testicular teratoma in this study have been reported to cause nausea, vomiting, mucosal ulceration and damage to the gastro-intestinal tract (Ohnuma and Holland, 1977; Donaldson and Lenon, 1979). Thus, treatment with these drugs caused a marked fall in body weight, plasma vitamin A and RBP, and to a lesser extent prealbumin. Treatment with these drugs also reduced the status of the patients with respect to other vitamins, including vitamin E, thiamine and pyridoxine.

In contrast to vitamin A, which is stored in the liver, thiamine and pyridoxine are not stored in appreciable amounts in the body, and the maintenance of blood levels is therefore dependent on normal dietary intake and absorption to a greater extent. It is noteworthy, that during treatment, the status with respect to the water-soluble vitamins either remained the same or deteriorated still further. In contrast, the plasma vitamin A, RBP and prealbumin concentrations rose to values higher than the pretreatment values at the start of the second and subsequent courses of chemotherapy. A greater increase was observed with the carrier proteins than with vitamin A. This could possibly be due to the reduction of some distant adverse effect of the neoplastic disease on the liver, for this organ plays a key role in the storage and mobilisation of vitamin A. Moreover, the elevation of plasma vitamin A seemed to be of prognostic value, as the vitamin A status did not improve during treatment when the disease progressed. It is of interest that, Soukop and Calman (1978) have also suggested that the measurement of plasma vitamin A concentration
might be of prognostic value.

The adverse effects of the drugs used in our patients were not restricted to cancer patients, but were also observed in normal healthy experimental animals. Thus, treatment of healthy male rats with vinblastine and bleomycin, either singly, or in combination, in doses comparable to those of teratoma patients, resulted in a significant reduction in food intake and body weight gain and caused depletion of vitamin A from the plasma and the liver. The toxic effects of vinblastine administered singly, or in combination, were greater than those of bleomycin, and vinblastine-treated animals had a dramatic loss in body weight. It is of considerable potential clinical importance that treatment with retinol (vitamin A), three hours prior to vinblastine administration markedly alleviated the toxic effects of the latter. Vitamin A-pretreatment caused a significant increase in food intake and curtailed the loss in body weight. Furthermore, it also prevented the fall in the concentration of total protein and albumin in the plasma, and the fall in the concentration of vitamin A in the plasma and liver. Thus, it seems reasonable to speculate that a similar beneficial effect might be exerted in cancer patients if treated with these or other antineoplastic drugs. It would be of interest also to ascertain whether vitamin A has the potential to alleviate the adverse effects of radiation therapy too.

However, the body's natural mechanism of averting vitamin A deficiency by storing excessive amounts in the liver confers a major potential limitation to vitamin A therapy. Thus, a dose response relationship was not observed on administration of high doses and
large amounts of vitamin A tended to be stored in the liver. Moreover, excessive amounts of retinoids in circulation cause undesirable toxic side effects (Smith and Goodman, 1976) although such effects were not observed in our study. It is of paramount importance therefore, to determine whether synthetic retinoids that have the ability to prevent or arrest certain types of tumour growth have the potential to alleviate the toxic effect of antineoplastic drugs.

A notable example is 13-cis retinoic acid, which has undergone extensive testing to evaluate its ability to arrest tumour promotion and progression (Sporn, 1977). This compound has been effective against a number of animal models which provide a spectrum of both transitional cell and squamous cell carcinoma of the bladder, which closely resemble the various stages of human disease (Sporn et al., 1977; Squire et al., 1977; Grubbs et al., 1977). In fact, these studies have prompted the start of a trial for the prevention of bladder cancer in disease-free patients who have undergone previous removal of at least two bladder carcinomas within six months prior to vitamin A therapy (Gunby, 1978). Several new synthetic analogues are now being evaluated in experimental studies (Sporn and Newton, 1979).

Recent studies have revealed the presence of intracellular binding proteins, cellular retinol-and retinoic acid-binding proteins, which might act as mediators of vitamin A action within the cell (Chytil and Ong, 1978; Chytil and Ong, 1979). The presence of these proteins could be an indication of whether the tumour responds to vitamin A therapy. The binding of retinoids to cellular binding proteins from experimental tumours correlated with their ability to
reverse metaplasias (Ong and Chytil, 1976; Chytil and Ong, 1978). Some tumours may contain both proteins, while some contain none or only cellular retinoic-acid binding protein (Chytil and Ong, 1979). An interesting point was the observation of cellular retinoic acid-binding protein in human lung and breast carcinomas, but not in the adjacent histologically normal tissue (Ong et al., 1975). Furthermore, these workers also observed a complete disappearance of cellular retinol-binding protein when compared to the healthy portion of the lung. However, the precise implications of these observations is not clear at present.

From the work presented in this thesis it seems possible to suggest that vitamin A plays a key role in carcinogenesis and in the treatment of cancer. Deficiency of this vitamin caused by a number of factors (Fig. 6.2), would tend to increase the incidence of spontaneous, carcinogen- or virus-induced epithelial metaplasias and tumours by virtue of effects on cell differentiation, on the metabolism of potential carcinogens, on susceptibility to carcinogens and also on immunocompetence. Consequent growth of a tumour would then cause systemic changes in the host which would further depress the vitamin A status. For example, as observed in patients with lung cancer, an increased requirement for zinc during growth of the tumour might cause zinc deficiency and in turn affect the mobilisation of vitamin A. Or, as in patients with metastatic testicular teratoma, impaired mobilisation of vitamin A might be a manifestation of a distant effect of the tumour on the liver. These deficiencies could be exacerbated by treatment such as chemotherapy and possibly radiotherapy. Moreover, low vitamin A status could increase the risk of infection and further complicate the disease process. Thus,
Fig. 6.2. Some possible interactions of vitamin A metabolism with tumour growth.
a deficiency of vitamin A might well be the key to a vicious circle (Fig. 6.2) affecting tumour incidence and growth and well-being of the host.

The interaction of vitamin A deficiency with tumour growth in a vicious circle could be interrupted to some extent by giving vitamin A or synthetic retinoids. Studies on cancer patients and follow up studies on experimental animals seem to suggest that treatment with vitamin A might enhance the body's natural defence mechanism and prevent the genesis or spread of the disease, particularly in patients with primary lung cancer. It may also alleviate the devastating effects of chemotherapy in disseminated malignant disease such as testicular teratoma. These studies have clearly demonstrated the potential role of a single nutrient in relation to both the genesis and progress of a particular tumour, such as lung cancer and of its potential as an adjuvant to the treatment of tumours.


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