THE EFFECT OF 3,5,3'-Triiodothyronine (T_3) ON THE EARLY STAGES OF COLON CARCINOGENESIS AND TISSUE LIPID COMPOSITION

by

GABRIELLA AUGELLONE

A Thesis
submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree of

MASTER OF SCIENCE

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ABSTRACT

Thyroid hormones exert a wide range of physiological responses in most mammalian tissues. 3,5,3'-triiodothyronine (T_3), the biologically active form, may induce a variety of effects in a species- and organ-specific manner. Besides its well known function as a regulator of metabolic processes, T_3 plays an important role in normal growth and development. Evidence that thyroid hormones may also affect neoplastic growth comes from laboratory based studies that have demonstrated enhanced initiation and progression of cancer by thyroid hormone treatment. The main objective of this research was to investigate the possible cancer modulating effect of 3,5,3'-triiodothyronine (T_3) in the whole organism. Aberrant crypt foci (ACF), precursor lesions of colon cancer, were used as the biological end point. A series of experiments was conducted to examine the effect of short term continuous T_3 feeding on the induction and growth of ACF, as well as on the proliferative features of the colonic epithelium of female CF1 mice and Sprague-Dawley rats. Additionally, experiments were conducted to assess the effect of continuous administration of T_3 on the lipid pattern and distribution in the liver and colonic mucosa of rats to gain insight into possible changes occurring at the tissue level. The animals were injected with the colon specific carcinogen, azoxymethane (5-10 mg/kg). T_3 was administered through diet at various levels (0.1 - 2.0

ppm) either prior to, during or following carcinogen treatment. The results of the experiments demonstrated that T_3 exerts a dose dependent increase on the induction and crypt multiplicity of ACF in the murine colon when administered in conjunction with the carcinogen, and increases cell proliferation in colonic crypts of mice. The number of ACF per colon for 0, 0.5, 1.0 and 2.0 ppm T_3 were 4.6, 10.6, 12.4 and 19.8, respectively. In the rat colon, T_3 did not alter the number and growth of ACF but did induce hyperplasia in the epithelial cells. It was also demonstrated that T_3 induces changes in the plasma and cell lipid composition in rat. These changes were characterised by a reduction in serum cholesterol concentration and a marked increase in the level of phosphotidylcholine in both the liver and colonic tissues. Furthermore, elevated levels of arachidonic acid were observed in both phosphotidylcholine and phosphotidylethanolamine. The findings of these experiments indicate that the modulating effect of T_3 on ACF is exerted in a species specific manner. CF1 mice were sensitive to this effect while Sprague-Dawley rats were not. The fact that T_3 stimulated a hyperplastic or anabolic response in the colon of both species suggests that unbalanced cell production may not be the main mechanism by which T_3 modulates the formation of ACF. This research provides an experimental framework with respect to the use of rodent models in studying the role of thyroid hormones in carcinogenesis.

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LIST OF ABBREVIATIONS

AC	aberrant crypt(s)
ACF	aberrant crypt focus (foci)
AOM	azoxymethane
BUdR	bromodeoxyuridine
b.w.	body weight
CH	crypt height
H & E	hematoxylin and eosin
i.p.	intra peritoneal
LC	labelled cells
MF	mitotic figures
PC	phosphotidylcholine
PE	phosphotidylethanolamine
PL	phospholipid(s)
ppm	parts per million
s.c.	sub cutaneous
SEM	standard error of the mean
TG	triglyceride
T_3	3,5,3'-triiodothyronine
T_4	3,5,3',5'-tetraiodothyronine, thyroxine

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PUBLISHED MATERIAL

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Augellone, G. and Bird, R.P. Effect of triiodothyronine (T_3) on aberrant crypt foci (ACF) formation and liver lipid composition in sprague-dawley rats (abs). Canadian Federation of Biological Societies conference. 1992.

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Chapter I

INTRODUCTION

Carcinogenesis is a complex multistage process which can be influenced by a variety of endogenous and exogenous factors. In recent years, considerable attention has been directed towards the cancer modulating effect of diet in various organs. Although dietary intake and composition have been shown to alter the incidence of cancer development and tumour growth, the underlying mechanisms remain poorly defined. It has been reported that hypercaloric consumption enhances the growth of various cancers in animal models while hypocaloric consumption retards tumour growth. A well known physiological effect of over- and undernutrition is to respectively increase and decrease blood levels of the biologically active thyroid hormone, 3,5,3'-triiodothyronine (T_3). Dietary intake and composition may also alter the metabolism and/or physiological potency of thyroid hormones.

Thyroid hormones exert a wide variety of biological responses in a species-specific and tissue-specific manner. Besides their well known function as metabolic regulators, thyroid hormones play an important role in growth and differentiation, and may therefore affect carcinogenic events. Various studies have demonstrated that the initiation and progression of cancer are enhanced by thyroid hormone treatment.

In view of the above mentioned effect of diet on thyroid status and biological activity, as well as the reported physiological action of thyroid hormone as a metabolic and growth regulator, it is hypothesised that thyroid hormones, particularly T_3 play a significant role in carcinogenesis.

Although thyroid hormones have been demonstrated to influence carcinogenic processes, most of the evidence has been observed in *in vitro* systems or in whole organisms harbouring transplanted cancer cells. A systematic evaluation of the potential role of thyroid hormone (T_4 and/or T_3) in the stepwise development of cancer of a specific site is lacking.

Nevertheless, investigation into the possibility that T_3 can affect carcinogenic events in various organs, including the colon, is an attractive one in view of the following properties of thyroid hormones.

(1) Thyroid hormones are ubiquitous hormones.
(2) Blood levels and biological potency of thyroid hormones vary from one individual to another, and are modulated by nutritional status and dietary factors.
(3) Thyroid hormones stimulate oxygen consumption, regulate the basal metabolic rate and influence most aspects of carbohydrate, lipid and protein metabolism.

(4) Thyroid hormones may affect the initiation and progressional stages of carcinogenesis.

(5) T_3 is capable of modulating the expression of various protooncogenes, the level of growth hormones and the responsiveness of an organ to growth factors.

(6) T_3 affects the activities of various membrane associated enzymes (Ca^{2+}-ATPase, Na^+-K^+-ATPase) and the permeability of the membrane to certain nutrients.

The main purpose of this dissertation was to assess the effect of T_3 on the early stages of colon carcinogenesis in the murine and rat models by enumerating the number and crypt multiplicity of ACF as biological endpoints. Furthermore, it was of interest to determine if chronic treatment of the animals with T_3 affects colonic cell proliferation and lipid composition in hepatic and colonic tissue.

Therefore, the main objectives of the present research were to:

(1) Quantify the possible modulating effect of elevated T_3 levels on early stages of colon carcinogenesis in rodent models (CF1 mice and Sprague-Dawley rats) and to assess the effect of T_3 on the proliferative status of colonic epithelia as a potential risk marker.

(2) Determine the effect of elevated levels of T_3 on the lipid composition of liver and colonic mucosa.

Chapter II

LITERATURE REVIEW

In order to put this dissertation into proper perspective a review of literature pertaining to the study of colon carcinogenesis, the relationship of diet to cancer development, and the physiological significance of thyroid hormones in normal and carcinogenic processes is provided.

A. Introduction to Cancer

Cancer development is a stepwise process characterized by the repeated selection of rare altered cell populations ultimately leading to the growth of an abnormal mass of malignant tissue (Farber, 1984). Three distinct stages of cancer development have been defined: initiation, promotion, and progression (Farber, 1984). The first stage, initiation occurs when the genetic component of the cells of a tissue are altered by a carcinogen (chemical, virus, radiation). Promotion involves the selective expansion of initiated cells into focal proliferations (nodules, papillomas, or polyps). The final stage of cancer development, progression, is the process by which focal proliferations undergo slow progressive growth resulting in malignant neoplastic cells. It should be noted that these three stages - initiation, promotion, and progression - are themselves the result of many steps (Farber

and Cameron, 1980).

B. Colon Cancer

1. Introduction

Colon carcinoma is one of the most prevalent forms of neoplasia affecting both men and women. An understanding of the pathogenesis of this disease and the risk factors associated with its development could significantly improve the incidence and/or mortality rate. Thus considerable attention has been directed towards determining the causes of colon cancer with the hope that such knowledge will lead to practical means of intervention.

The genesis and progression of colon cancer is complex. The evolution of normal mucosa into cancer of the colon is a multistep process which proceeds through abnormal mucosal proliferation to adenoma formation, growth, and malignant transformation (Boone et al., 1992). Multifactorial forces are responsible for the sequential transformation of normal colonic mucosa to cancer (Winawer and Shike, 1992). However, the evidence is strongest for a substantial environmental contribution to the risk of colon cancer (Willet, 1989). In particular, the origin of cancer of the colon appears to be associated with diet and nutritional factors (Bruce, 1987). It has been estimated that dietary changes could prevent up to

90% of the cases of colon cancer (Doll and Peto, 1981). This indicates the importance of studying the role of diet and specific dietary risk factors in the disease process.

2. Experimental Colon Carcinogenesis

The colon represents a favourable organ for the experimental study of carcinogenesis (Bruce, 1987). All of the stages involved in colon cancer development can be explored, ranging from early biomarkers to tumorigenesis.

Two common approaches for investigating the effect of various dietary factors in the development of colon cancer are induction of tumours involving a large number of animals and a long term study (30-40 weeks), and risk markers as biological endpoints involving fewer animals and a shorter duration experiment (2-12 weeks) (Bird et al., 1989). Tumorigenesis studies provide a measure of the actual disease, but this is not a suitable method for assessing the role of diet in the multiple steps leading to the disease. The limitation of utilizing risk markers as endpoints is that they do not represent the disease itself but instead provide an indication of risk of developing the disease. Validation of biomarkers in order to reliably predict the modulation of cancer risk is lacking and this emphasizes the need to establish a system which allows quantitative assessment of the stepwise development of colon cancer.

3. Aberrant Crypt Foci

Recently, Bird (1987) described a method of identifying and quantifying abnormal crypts, termed aberrant crypts (AC) in the colons of rodents treated with a colon carcinogen. She hypothesized that these foci of aberrant crypts (AC) represent early precursor lesions of colon cancer. Further investigations supported this novel discovery (Bird et al., 1989).

It has been demonstrated that aberrant crypts (AC) are stable focal lesions possessing atypical morphological characteristics (McLellan et al., 1991b). They are induced specifically by colon carcinogens in a dose dependent and species specific manner (McLellan et al., 1991a; McLellan and Bird, 1991; McLellan and Bird, 1988a,b; Tudek et al., 1989). They grow in size and their induction and growth can be modified by diet (Caderni et al., 1990; Corpet et al., 1990; McLellan and Bird, 1988b). In addition, the induction of AC has been shown to be inhibited by well known inhibitors of colon cancer (Lam and Zhang, 1991; McLellan and Bird, 1991; Pereira and Khoury, 1991) further supporting the theory that AC represent precursor lesions of colon cancer. AC have recently been identified in human colonic tissue (Pretlow et al., 1991; Roncucci et al., 1991).

The AC assay represents a simple yet sensitive approach for

studying early preneoplastic changes in the colon and the stepwise assessment of the disease development. The entire mucosal surface can be examined using light microscopy to identify abnormal crypts, or aberrant crypt foci (AC). AC are distinguished from normal crypts by their increased size, elongated shape, and more prominent epithelial cells (Bird, 1987). This system allows direct identification and quantification of carcinogen induced preneoplastic lesions in the colon of rodents. Number, size and crypt multiplicity (number of crypts per focus) of AC can be determined. Even a single focus can be quantified in a colon consisting of innumerable crypts.

C. Diet and Cancer

1. Introduction

Epidemiological, experimental and clinical investigations have implicated diet as an important causative agent in the aetiology of colon cancer (Reddy et al., 1980). Initial investigations into the role of diet in the causation of large bowel cancer generally focused on the effect of macronutrients, and specifically on the detrimental and protective effects of fat and fibre, respectively (Wargovich et al., 1988; Willet, 1989; Willet and MacMahon, 1984; Winawer, and Shike, 1992). Recently, Giovanucci et al. (1992) supported the hypothesis that a diet high in saturated fat and

low in fibre increases the risk of colorectal carcinoma. It is generally believed that the relationship between dietary factors and colon cancer is associated with alterations in cell proliferation (Jacobs,1988). Thus factors that stimulate and reduce cell proliferation will respectively enhance and inhibit tumour development. Current research into the causative and protective role of dietary factors in carcinogenesis has assigned importance to micronutrients, including vitamins and minerals such as vitamins A and C, calcium and selenium (Wargovich, 1988; Willet and MacMahon, 1984).

While the majority of studies investigating the diet-cancer relationship have focused on the effect of specific macro- and micronutrients, little attention has been directed towards the role of total energy and nutrient intake in colon carcinogenesis. Lyon et al. (1987) pointed out that the daily intakes of the energy yielding nutrients are highly correlated and cannot be tested in isolation when examining the role of diet in disease causation. Thus, it is important to consider the influence of total food or energy intake on cancer risk.

2. Caloric Intake and Cancer Risk

Caloric intake can affect the risk of developing cancer. Caloric restriction appears to be one of the most effective ways of reducing cancer risk and increasing longevity in

experimental animals (Pariza, 1987). Tumour incidence decreases and the growth of transplantable tumours is retarded by underfeeding. The possibility that limiting energy intake may reduce cancer risk in humans requires further investigation.

In contrast to the protective effects of undernutrition, caloric excess has been implicated as a risk factor for cancer development (Willet and MacMahon, 1984). Ad libitum feeding in rodents increases the risk of tumour development (Pariza, 1987). Human studies also suggest a relationship between overnutrition and cancer risk. In a case-control study, substantially higher levels of caloric intake was reported for subjects with cancer of the colon as compared to controls (Lyon et al., 1987). A recent study found that the risk of developing colon cancer as measured by incidence of colorectal adenomatous polyps increased with increasing body mass index (Neugut et al., 1991). Such findings cannot rule out the possibility that increased ingestion of calories may in fact be the true risk factors (West et al., 1989).

3. Mechanism of action of the caloric effect

The mechanism of action of hyper- and hypocaloric nutrition on cancer risk is not well understood. It has been postulated that alterations in hormone status which accompany changes in dietary intake and composition may play a vital role in the

carcinogenic process (Pariza, 1987). Hence, the hormonal changes resulting from caloric excess and caloric restriction may increase and decrease tumour risk respectively.

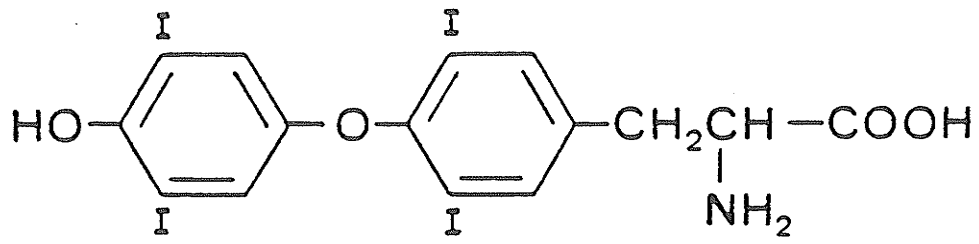
Hormones and growth factors are speculated to play a significant role in the genesis and progression of the neoplastic process in the colon (Conteas et al., 1988). However, to date little is known about the importance of various hormones and their interrelationship between diet and cancer.

D. THYROID HORMONES

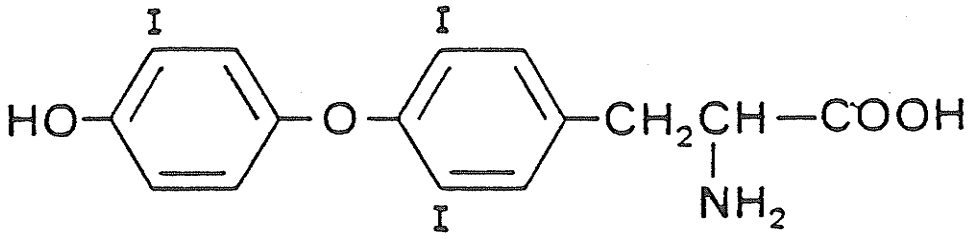
1. Biosynthesis and Secretion

The biosynthesis and secretion of thyroid hormones has been described in detail by Granner (1988). The thyroid gland produces 2 iodoamino acid hormones, 3,5,3'-triiodothyronine (T_3) and 3,5,3',5'- tetraiodothyronine (T_4, thyroxine) (Figure 1). Thyroid hormones are unique in that they require the trace element iodine for biological activity. The biosynthesis of the thyroid hormones involves four sequential stages of iodine metabolism: active transport of iodide into the thyroid follicular cells, oxidation of iodide, iodination of tyrosyl residues and coupling of the iodotyrosines to form the active hormones, T_4 and T_3. The thyroid hormones are stored in the follicular lumen incorporated into the precursor

Fig. 1. Structure of the thyroid hormones.



3,5,3′,5′-Tetraiodothyronine (thyroxine [T_4])



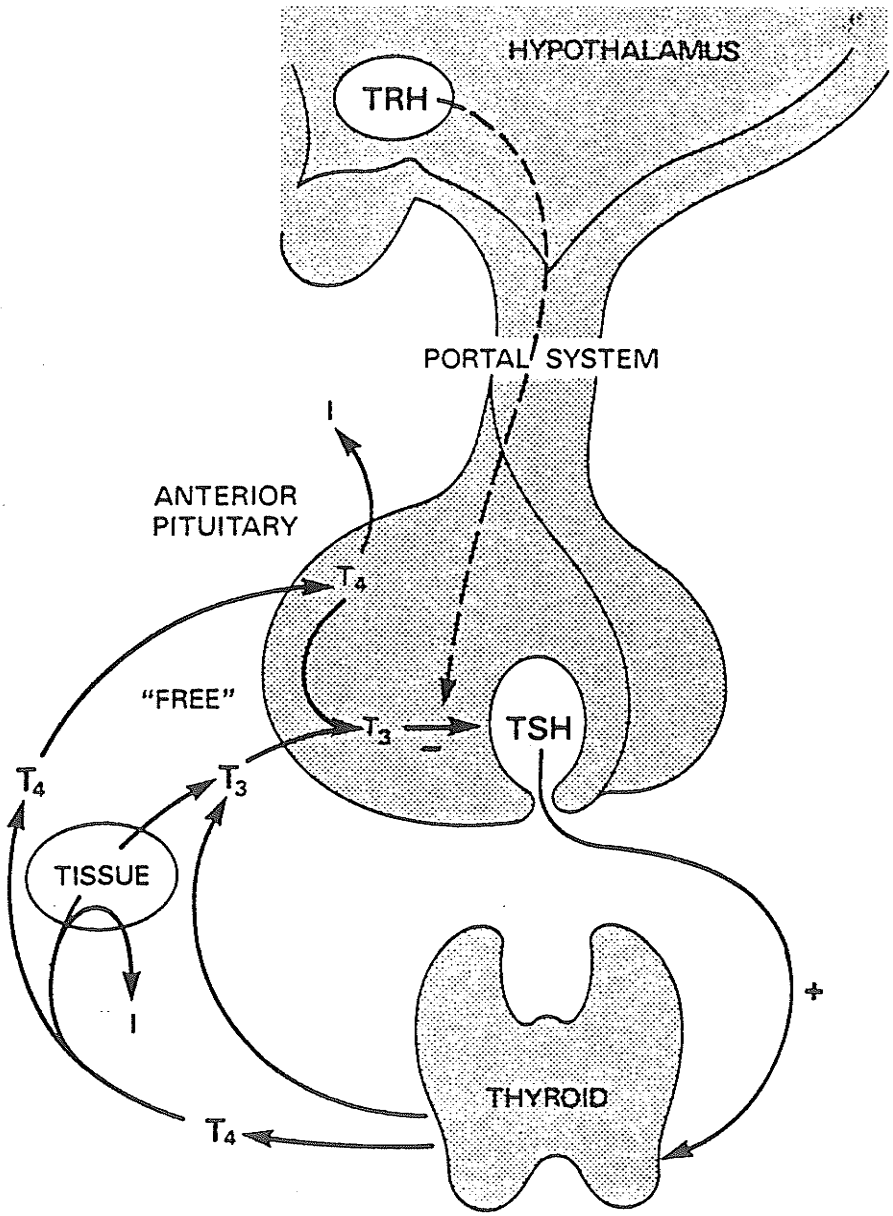
3,5,3′-Triiodothyronine (T_3)

molecule, thyroglobulin. Upon stimulation of the thyroid gland, thyroglobulin is taken up again by the follicular cells and hydrolysed by proteases and peptidases, releasing the iodothyronines which are discharged into the bloodstream. The iodotyrosines undergo intrathyroidal deiodination via the enzyme deiodinase and the resulting free iodide is reutilized.

Regulation of thyroid hormone biosynthesis and secretion involves a negative feedback regulatory system (Granner, 1988; Mazzaferri, 1985). Free T_3 and T_4 cause feedback inhibition of their own synthesis by inhibiting the release of thyroid stimulating hormone (TSH) from the pituitary and perhaps also the release of thyroid releasing hormone (TRH) from the hypothalamus which in turn stimulates TSH release. The interaction between the hypothalamic, pituitary, and thyroid axis is depicted in Figure 2. The stimulus for increased release of TRH and TSH is a decrease of free thyroid hormones in the blood. There is also an interesting feedback loop between thyroid and growth hormone. T_3 and T_4 enhance the release of somatostatin (SRIH) from the hypothalamus, which in turn inhibits TSH release from the pituitary. Levels of SRIH can also increase in response to increased plasma insulinlike growth factor I (IGF-I), which in turn is stimulated by growth hormone.

2. Transport and Metabolism

Fig. 2. Feedback regulation of thyroid hormone biosynthesis. (Taken from Greenspan and Rapoport, 1991)



A wide variety of iodothyronines and their metabolic derivatives exist in plasma. T_4, which is derived solely from secretion from the thyroid gland, is present in the highest concentration. T_3 in the blood is partly derived directly from the thyroid gland (less than 20%), but the majority (80-90%) is generated from the outer ring monodeiodination of T_4 in the peripheral tissues. Among the various other iodothyronines found in plasma is 3,3'5'-triiodothyronine or reverse T_3 (rT_3), a relatively inactive hormone formed from inner ring deiodination of T_4. Figure 3 quantitatively outlines the thyroidal secretion of T_4, T_3 and rT_3, and the peripheral conversion of T_4 to T_3 and rT_3.

More than 99% of the thyroid hormones circulates in the blood bound to three specific binding proteins: thyroxine-binding globulin (TBG), thyroxine-binding prealbumin (TBPA), or albumin (Figure 4). Quantitatively, TBG is most important as it accounts for the majority of the total T_3 and T_4 bound to protein. A lesser amount of T_4 and a negligible amount of T_3 are bound to TBPA. The remaining is primarily bound to albumin. The small unbound fraction (free T_3 and free T_4) is responsible for the biological activity of thyroid hormone. T_3 binds to specific receptors with about 10 times the affinity of T_4 and is therefore considered the predominant metabolically active form (Oppenheimer, 1975).

Fig. 3. Thyroidal secretion and peripheral metabolism of thyroid hormones. (Taken from Mazzaferri, 1985)

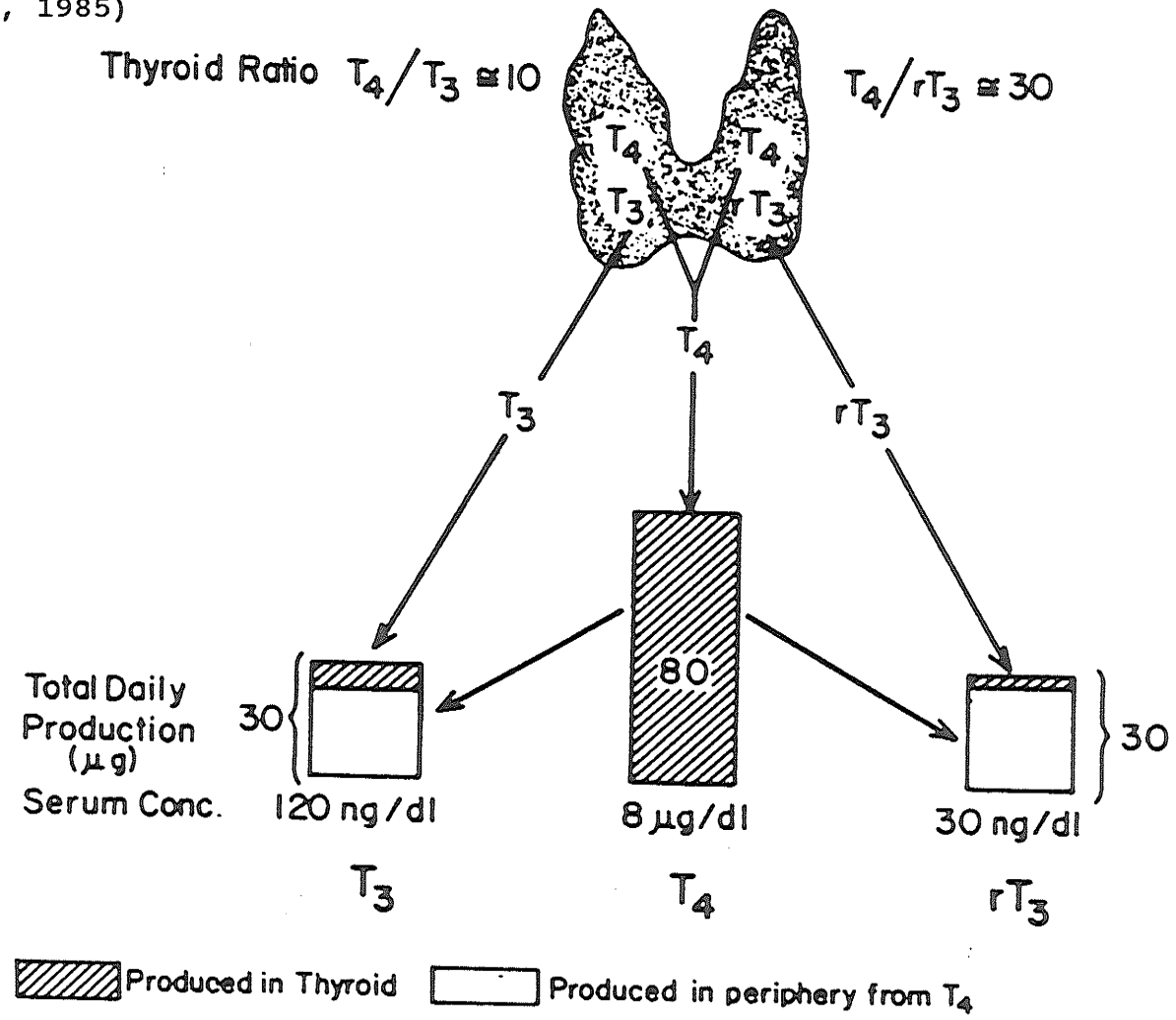
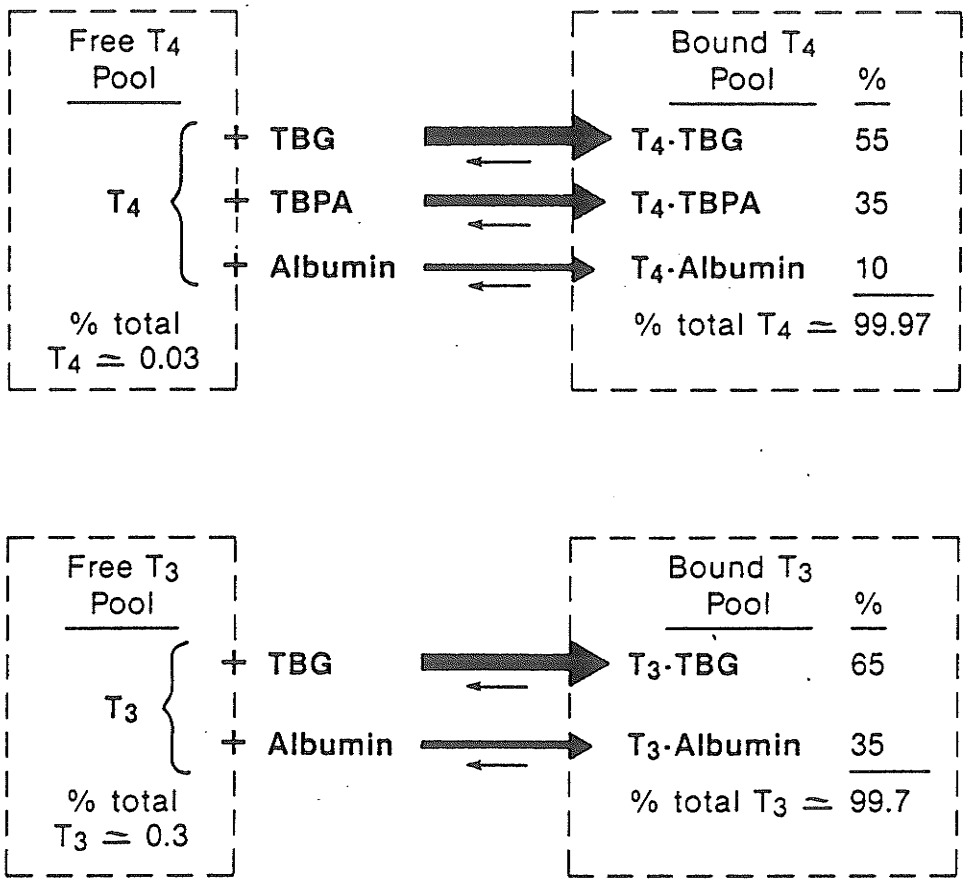


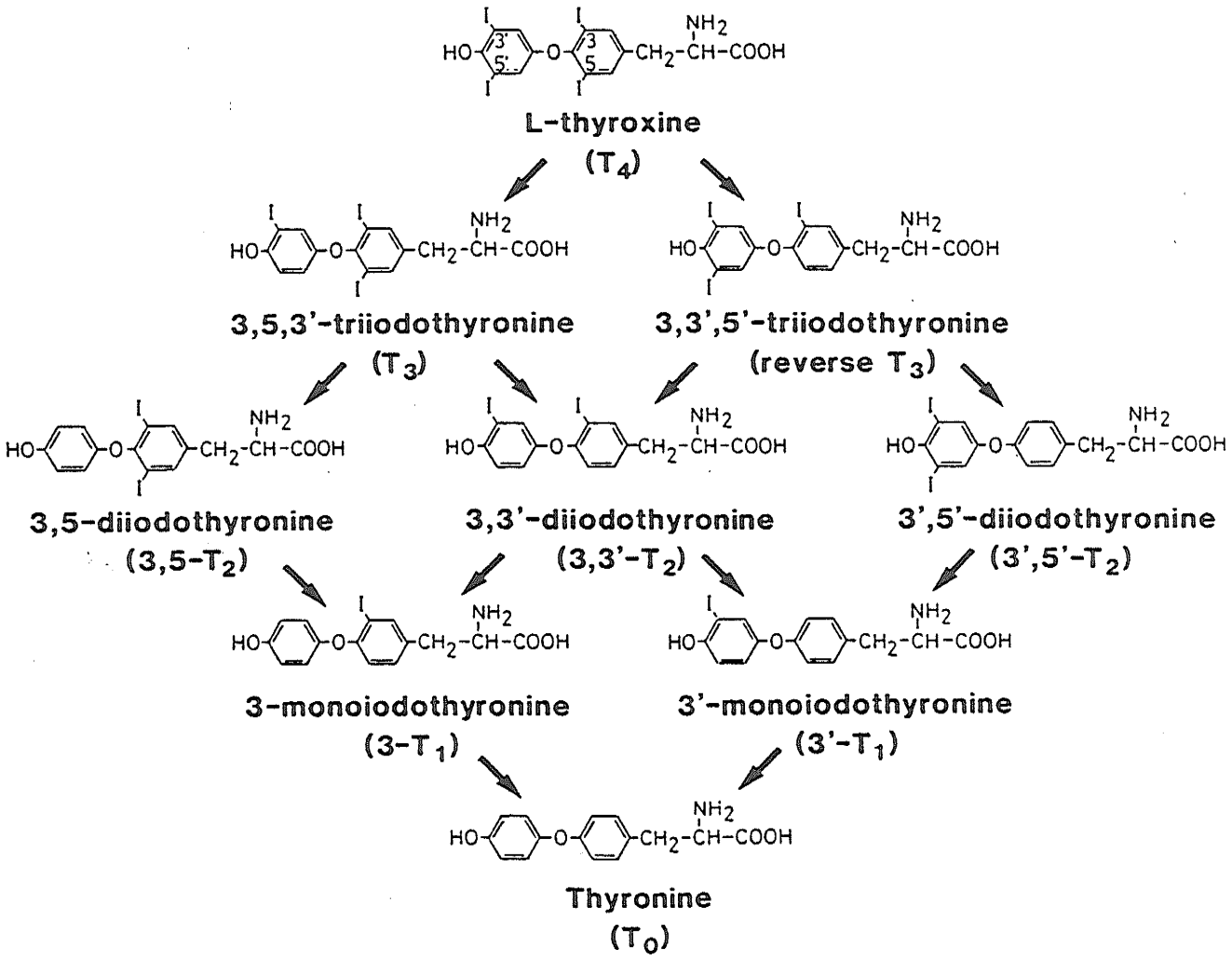
Fig. 4. Binding of T_3 and T_4 to plasma proteins. (Taken from Hedge et al., 1987)



The metabolic fate of circulating thyroid hormones has been reviewed in detail by Engler and Burger (1984). Complete metabolism of T_4, once it is secreted from the thyroid gland, involves a series of complex and inter-connected steps including deiodination, deamination and decarboxylation of the amino acid side chain, and esterification of the phenolic hydroxyl group with glucuronic acid or sulfuric acid. Deiodination of T_4 is the main degradative pathway accounting for as much as 85% of the hormone's disposal (Figure 5). This enzymatic, reductive process involves the specific removal of iodine atoms from both rings of the molecule by the action of deiodinases, and their replacement by hydrogen atoms. T_3 and rT_3 are generated from 5'- and 5-deiodination of T_4, respectively. These triiodothyronines are further sequentially deiodinated to yield diiodothyronines, monoiodothyroinines and ultimately thyronine.

Although progressive deiodination is the major disposing pathway, there are other minor metabolic conversions (Greenspan and Rapoport, 1991). T_4 and T_3 can undergo oxidative deamination yielding tetraiodothyroacetic acid (Tetrac) and triiodothyroacetic acid (Triac), respectively. Circulating iodothyronines can be conjugated in the liver with glucuronic acid and excreted in bile, where they may then reenter the bloodstream through the enterohepatic circulation, or they can be conjugated in the kidney with sulfate and

Fig. 5. Metabolism of thyroid hormones by monodeiodination. (Taken from Engler and Burger, 1984)



excreted in urine. Thyroxine can also be decarboxylated to thyroxamine. In addition, the ether bond between the two rings of the iodothyronines can be cleaved producing diiodotyrosine and iodide (Engler and Burger, 1984).

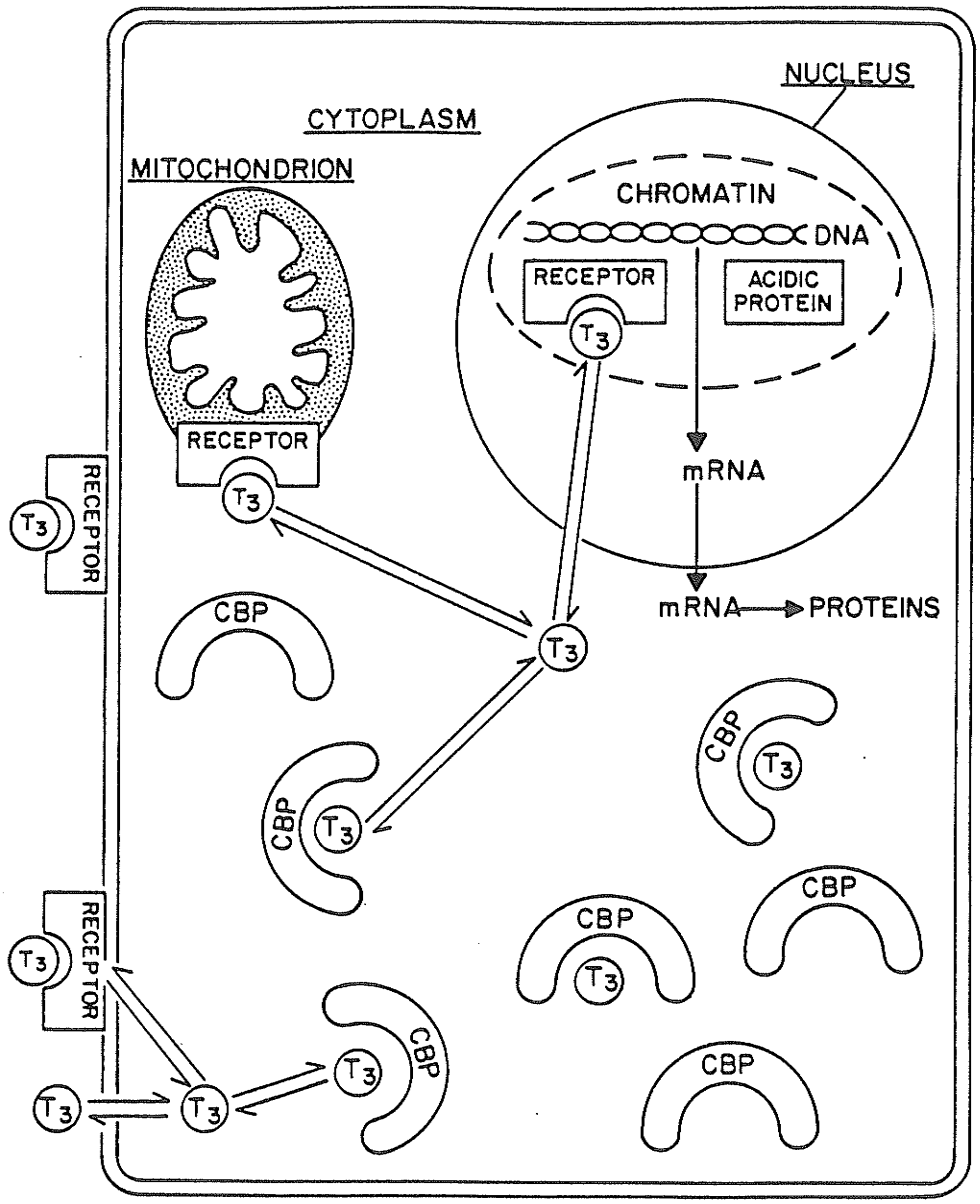
3. Mechanism of Action

Although significant progress has been made towards understanding the mechanism of action of thyroid hormones, their properties are still poorly understood partly because of a lack of a well defined set of target sites (Oppenheimer, 1979). Thus, the precise events responsible for the multitude of actions exerted by thyroid hormones has been difficult to elucidate due to the wide variety of physiological responses.

(a) Genomic

Figure 6 illustrates a common model for thyroid hormone action on the target cell. The sequence of events is believed to be similar for T_4 and T_3 (Sterling, 1979). Unbound T_3 diffuses or is transported by plasma receptors into the cell where it is bound by cytosolic-binding protein or proteins (CBP). The presence of cytoplasmic binding sites may serve to keep T_3 in the target cells. The CBP-T_3 complex exists in a reversible equilibrium with a minute moiety of free T_3. It is this free fraction which binds to thyroid hormone receptor proteins. It is believed that the specific binding of T_3 to low capacity, high affinity nuclear receptors leads to alterations in the

Fig. 6. Model for thyroid hormone action on the target cell. (Taken from Sterling, 1989)



expression of certain genes (DeGroot, 1990; Oppenheimer, 1975; Oppenheimer, 1979; Oppenheimer, 1985; Sterling, 1979). Hence, thyroid hormones initiate action at the nuclear site by regulating the level of specific mRNA's. These mRNA's are translated into proteins whose subsequent structural or enzymatic function represents the physiological responses exerted by thyroid hormones. The nuclear T_3 receptor has been identified in various rat tissues (liver, kidney, pituitary, heart, brain, spleen and testis) (Oppenheimer et al., 1974) and has been characterized as a non-histone protein tightly associated with chromatin (Surks et al., 1973). The molecular mechanisms by which thyroid hormones mediate their effect on gene transcription and protein synthesis are currently unclear. Possible stimulation of the translation process by thyroid hormone has been suggested but as yet requires further investigation (Sterling, 1979).

(b) Nongenomic

Although there is strong evidence for the presence of nuclear T_3 receptor sites, the mediation of thyroid hormone action through independent extranuclear pathways cannot be excluded. The presence of T_3 receptors in mitochondrial membranes has been repeatedly postulated although findings up to date are not consistent (Dillmann, 1985). Support for mitochondrial T_3 receptors is primarily based on the well established site of oxidative phosphorylation, which is accelerated by thyroid

hormone administration (Sterling, 1979).

Evidence is also mounting for a direct action of thyroid hormone at the level of the plasma membrane (Segal, 1989). Several tissues display specific T_3 binding to plasma membrane sites and in some cases the T_3-receptor complexes have been linked to specific thyroid hormone effects (Dillmann, 1985). Various studies have demonstrated stimulation of cell membrane Ca^{2+} ATPase by physiological levels of thyroid hormone *in vitro* (Segal, 1990; Davis et al., 1989). Thyroid hormones also stimulate the plasma membrane enzyme Na^{+}-K^{+}-ATPase, the "sodium pump", which is responsible for increased oxygen consumption (Ismail-Beigi, 1992). It has further been shown that T_3 increases the entry of amino acids and sugars into the cell via an active transport mechanism independent of nuclear T_3 effects (Dillmann, 1985; Segal, 1989).

4. Physiological Functions

Thyroid hormones have ubiquitous effects and influence the function of most organs in various species. They have long been recognized for their importance in regulating general metabolism. Thyroid hormones exert a pronounced stimulatory effect on oxygen consumption which is a measure of the basal metabolic rate (Danforth, 1983; Acheson et al., 1984). This action is frequently mediated by increasing the levels and/or activity of specific enzymes which contribute to O_2

consumption, such as the Na^+-K^+-ATPase of the plasma membrane and mitochondrial α-glycerophosphate dehydrogenase (Berdanier and Shubeck, 1981; Lee and Lardy, 1965).

Thyroid hormones also regulate the metabolism of carbohydrate, lipid and protein. Virtually all aspects of energy metabolism are stimulated by thyroid hormones indicating an association with 'futile cycles' (Mariash and Oppenheimer, 1985). Enhanced glucose production and utilization, as well as accelerated lipid synthesis and oxidation accompany hyperthyroidism. Similarly, both synthesis and degradation of proteins are enhanced by thyroid hormones.

Besides their well known effect on energy metabolism, thyroid hormones play an important role in the complex biological processes involved in growth and differentiation. Thyroid hormones are necessary for normal growth, development and homeostasis in young and adult vertebrates. For example, the important role of thyroid hormones in the normal development of the brain and central nervous system has been recognized for a long time (Stein et al., 1990). It has been shown that maternal hypothyroidism in humans and rats results in a high incidence of behavioral and neurological fetal disorders (Porterfield and Hendrich, 1990).

A large body of evidence for the growth regulatory role of

these hormones comes from thyroid hormone induced increases in growth hormone (GH) synthesis. T_3 stimulates the accumulation of GH mRNA levels *in vivo* and in rat pituitary cell cultures, considered to be effective in studying TH action (Samuels et al., 1989; Spindler et al., 1989). Furthermore, thyroid hormones alter the levels of various growth factors in mice. Adequate production of nerve growth factor (NFG) and epidermal growth factor (EGF) in the mouse submaxillary gland (SMG) is regulated by thyroid hormones (Walker, 1982; Watson et al., 1982).

E. EFFECT OF DIET ON THYROID STATUS

The interrelationships between dietary factors and thyroid function is unquestionably complex. Alterations in quantity or pattern of food consumption influence thyroid function, directly or indirectly, at levels ranging from neural control of thyroid stimulating hormone (TSH) release to postreceptor cellular action of thyroid hormone (Eales, 1988). All three energy yielding macronutrients, carbohydrate, fat and protein have been implicated in this process in humans, with the bulk of research focusing on the role of carbohydrates (Burman et al., 1979; Macdonald, 1989; Young et al., 1982).

The concept that diet induced thermogenesis is mediated via increased levels of thyroid hormone, particularly T_3, is well

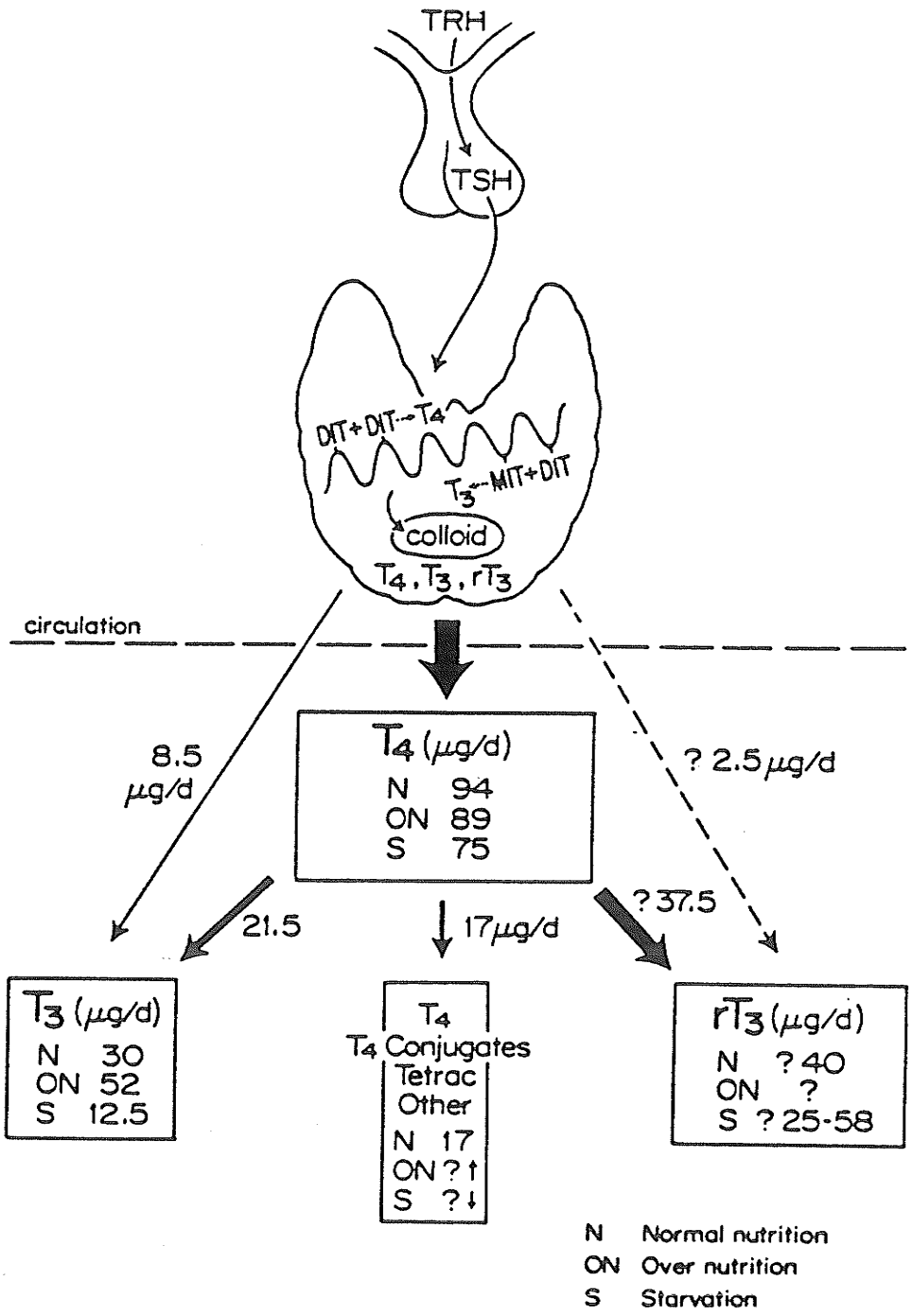
known (Acheson et al., 1984; Danforth, 1985;). Furthermore, overfeeding and underfeeding are associated with both central and peripheral alterations in thyroid hormone metabolism (Danforth, 1983). Figure 7 summarizes the recognized effect of over- and undernutrition on peripheral thyroid hormone metabolism.

1. Underfeeding

In humans, hypocaloric feeding causes blood concentrations of T_3 to fall due to a substantial reduction in peripheral T_4 to T_3 conversion, despite a decrease in T_3 metabolic clearance rate (MCR). Blood rT_3 concentration increases due to a decrease in rT_3 MCR. There is a modest drop in blood T_4 and T_4 MCR (Grant et al., 1978; Pasquali et al., 1982; Spaulding et al., 1976; Vagenakis et al., 1975; Visser et al., 1978). In addition, despite lower T_3 concentrations, the pituitary thyroid stimulating hormone (TSH) response to thyrotropin releasing hormone (TRH) is diminished (Hugues et al., 1984; O'Brian et al., 1980). Caloric restriction in rodents, and especially in rats, is associated with similar alterations in thyroid hormone metabolism as reported for humans except that blood T_4 is considerably lowered by hypocaloric nutrition (Balsam and Ingbar, 1978; Harris et al., 1978; Hugues et al., 1984).

2. Overfeeding

Fig. 7. Effect of over- and undernutrition on thyroid hormone metabolism. (Taken from Danforth, 1983)



Only a limited number of studies has investigated the relationship between thyroid hormones and hypercaloric nutrition and these have focused mainly on human responses. Generally, the metabolic changes reported for over-nutrition are opposite to those observed in underfeeding in humans: blood T_3 levels are increased due to enhanced T_4 to T_3 conversion; rT_3 levels are reduced; and T_4 levels, as well as its MC and production, remain unaltered (Danforth et al., 1979; Davidson and Chopra, 1979; Edozien, et al., 1978; Welle et al., 1986).

In addition to regulating the central and peripheral metabolism of T_4, T_3 and rT_3, dietary composition and intake may also affect the biological potency of thyroid hormone by altering the number of T_3 receptors in various target tissues. For example, starvation has been shown to reduce the number of hepatic nuclear receptors for T_3 in rats (Schussler and Orlando, 1978).

F. THYROID HORMONES AND CARCINOGENESIS

Hormones are known to have dramatic effects on carcinogenesis, affecting both the initiation of neoplastic transformation and the neoplastic phenotype (Conteas et al., 1988; Goustin et al., 1986). Evidence for the possible role of thyroid hormone in the carcinogenic process is derived mainly from laboratory

studies, as data from human epidemiological studies are limited and conflicting. In general, thyroid hormone treatment is associated with increased frequencies of some forms of neoplasia, enhanced growth of transplantable tumours and aggression of metastatic proliferation (Cave et al., 1977; Kumar et al., 1979). Hypothyroidism appears to reverse some of the these effects (Kellen, 1972, Kumar, 1979).

It is generally indicated that thyroid hormones serve as crucial factors in the initiation of cellular neoplastic transformation (Borek et al., 1985; Borek et al., 1983; Lopez et al., 1989). This suggests that the thyroidal status of the animal or cell culture system at the time of carcinogen exposure modulates the development of tumours and transformed cells, respectively. Various cell cultures are more vulnerable to transformation by activated Ha-ras oncogenes, viruses, radiation or chemicals in medium supplemented with T_3 as compared to T_3-depleted medium. Furthermore, thyroid hormone has been shown to enhance the expression of K-ras protooncogene in cells thereby rendering the cells more vulnerable to mutations and transformation (Guersney and Leuthauser, 1987).

The effect of thyroid hormone in the development of neoplastic disease has also been demonstrated *in vivo*. Recently, Iishi et al. (1992) found that T_4 treatment increased the incidence

of colonic tumours in rats given multiple injections of a colon carcinogen. It has also been demonstrated that thyroid hormone may play a role in the progressional stages of carcinogenesis. In animals made hyperthyroid by T_4 treatment, implanted cancer cells demonstrate a more aggressive pattern of tumour growth and metastasis as compared to hypothyroid counterparts which display retardation of tumour growth and spread (Kumar et al., 1979).

It is also interesting to note that nuclear T_3 receptors have been identified in human tumours of various origins (Lemaire and Baugnet-Mahieu, 1986). Among the tissues displaying T_3 receptor sites were breast cancer, central nervous system tumours, sarcomas and epitheliomas including stomach and colon cancer. The presence of T_3-binding sites in human cancer tissue further suggests that thyroid hormones may play a role in carcinogenesis.

Chapter III

MATERIALS AND METHODS

The materials and methods which were used in more than one experiment are listed below.

A. Animals

Two species of female animals were used. CF1 mice were purchased from Charles River Canada Inc., Montreal, Quebec. Sprague-Dawley rats were purchased from the Central Animal Breeding Facility, University of Manitoba. Mice were housed in stainless steel cages with sawdust bedding in groups of three or four. Rats were housed in stainless steel cages with wire mesh bottom in groups of two or three. All animals were maintained on a 12-h light:dark cycle. Food and water were provided *ad libitum*. All animals were cared for in accordance to the guidelines of the Canadian Council on Animal Care.

B. Diets

In all studies, animals were acclimatized for about one week on Purina Laboratory Chow, a natural ingredient diet made from ground corn, meat and bone meal, soybean meal, wheat middlings, ground wheat, ground oats, dehydrated alfalfa meal, dried milk product, brewers dried yeast, dried molasses, animal fat (with preservatives), iodized salt, dicalcium phosphate, and vitamin and mineral premixes. The composition

of the Purina Laboratory Chow is presented in Table 1. The experimental regime consisted of a powdered AIN-76 semi-synthetic diet (Report of the American Institute of Nutrition, 1984). The composition of the AIN-76 diet is listed in Table 1. (See Appendix A for details of the vitamin and mineral mixture). Test diets were formulated by adding 3,5,3'-triiodo-L-thyronine (T_3) (Sigma Chemical Co., St. Louis, MO) in various dosages (0.05, 0.1, 0.25, 0.5, 1.0, or 2.0 ppm) to the AIN-76 diet. This mode of T_3 treatment has been used previously in the murine system (Perry et al., 1988; Woods and Woodward, 1991). The required concentration of T_3 was initially dissolved in ethanol and mixed into 50 g of diet. The ethanol was allowed to evaporate in the fumehood and then the diet premix was added to the rest of the diet.

TABLE 1. Percentage by weight composition of the diets.

AIN-76		LAB CHOW	
Ingredient	%	Ingredient	%
sucrose	50.0	NFE*	44.20
casein	20.0	protein	25.21
dextrin	15.0	ash	10.62
corn oil	5.0	moisture	9.28
alpha-cellulose	5.0	fat	6.37
AIN-76 mineral mix	3.5	crude fibre	4.32
AIN-76 vitamin mix	1.0		
DL-methionine	0.3		
choline bitartrate	0.2		

* NFE = nitrogen free extract - calculated carbohydrate portion

C. Preparation of the Carcinogen

The colon specific carcinogen azoxymethane (AOM) (Sigma Chemical Co., St. Louis, MO) was used in all experiments. The required concentration of AOM was prepared fresh in a saline solution prior to its use.

D. Body Weights

Body weights (b.w.) of all animals were recorded at the start of the experiment and each week thereafter.

E. Food Consumption

The amount of food consumed per day was calculated by taking an average of the last three days of each week. Food cups were filled and weighed on the fifth day of each week and then weighed again at the same time every 24 hours for three days.

F. Preparation of the Colons

All animals were terminated by carbon dioxide asphyxiation. Upon termination, the colons were excised, flushed with phosphate buffered saline, slit open from caecum to anus, and fixed flat in phosphate buffered formalin between filter papers. Colons which were to be analyzed for the presence of labelled cells were fixed in 70% ethanol (see H - 2 below)

G. Identification and Quantification of Aberrant Crypt Foci

Colons were prepared and examined according to the method developed by Bird (1987). The colons were placed in a petri

dish and stained for 15-30 minutes with 0.2% methylene blue (Sigma Chemical Co., St. Louis, MO) dissolved in saline or Kreb's Ringer salt solution. The colons were then placed on a glass slide and the entire mucosal surface was examined under light microscopy at a magnification of 40x. Aberrant crypt foci (ACF) were distinguished from normal crypts by their increased size, elongated shape, more prominent epithelial lining, and larger pericryptal zone (the amount of area between bordering crypts) (McLellan and Bird, 1988). Typical ACF are shown in Figure 8. Number, size and crypt multiplicity (number of crypts per focus) of ACF were determined for each colon by scanning the entire mucosa beginning at the rectal end. The size of ACF was assessed by placing a grid in the eyepiece of the light microscope. The number of squares on the grid that the focus occupied was then determined under magnification 100x.

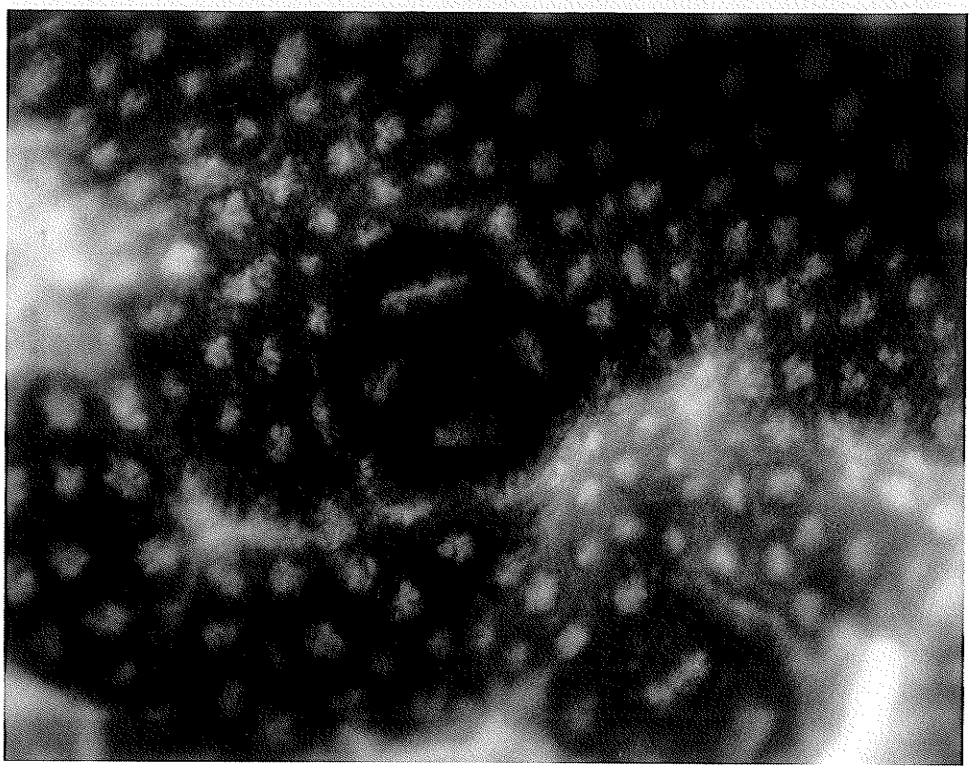
H. Assessment of Proliferative Characteristics

1. Crypt height

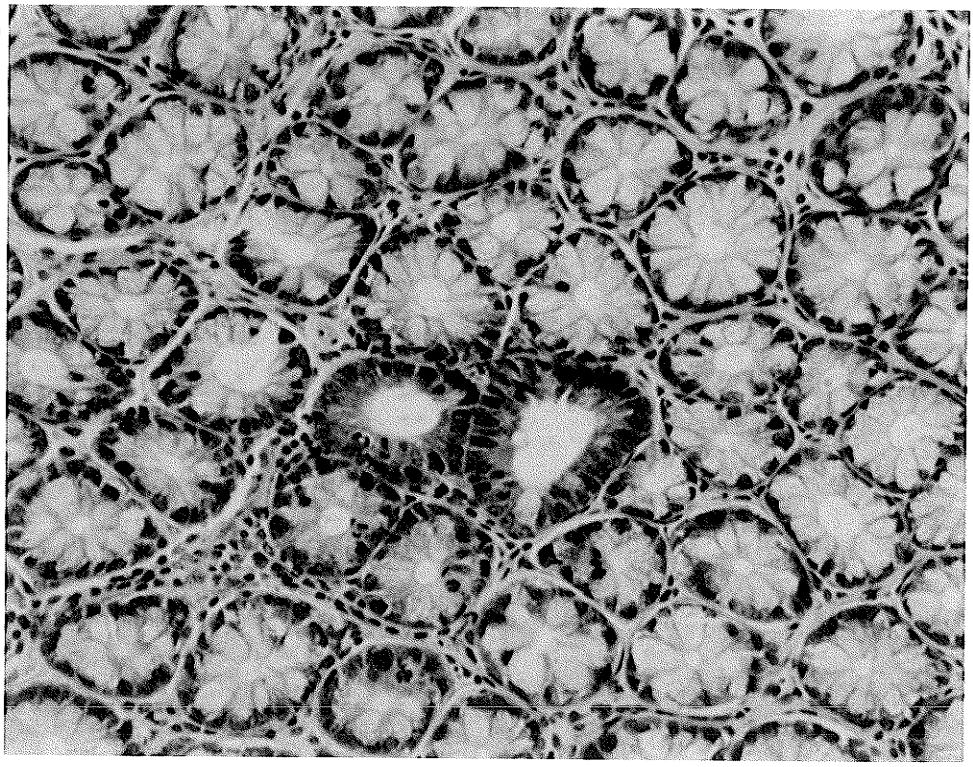
Tissues were embedded in paraffin, sectioned longitudinally and mounted on slides. Prior to staining, the sections were de-waxed and rehydrated. The slides were then dipped in haematoxylin for 15 seconds and washed in running tap water. Excess stain was removed by decolorizing with 0.25% HCl in 70% alcohol. The blue colour was regained by washing in 2% sodium hydrogen carbonate. The slides were then stained with 0.5%

Fig. 8. Topographical views of ACF consisting of various numbers of crypts. (a) Topographical view of unsectioned methylene blue-stained rat colon. The aberrant crypt foci are characterised by increased crypt size, luminal openings of varying shapes and increased epithelial lining. (b) Topographical view of a transverse section of rat colon. The aberrant crypt focus contains two crypts. Note that the nuclei of the ACF are elongated and hyperchromic.

A



B



eosin for five minutes, washed and again dehydrated with alcohol. Nuclei were depicted by their blue-black coloration. The height of colonic crypts was measured by counting the nuclei lining the length of the crypts.

2. Bromodeoxyuridine labelled cells

Number of labelled cells in colonic crypts was determined with an immunohistochemical analysis. Animals received a s.c. injection of bromodeoxyuridine (BUdR), 50 mg/kg b.w., (Sigma Chemical Co., St. Louis, MO) one hour prior to killing. The colon was removed and fixed in 70% ethanol for at least 24 hours. Tissues were embedded in paraffin, sectioned longitudinally and placed on poly-l-lysine coated slides. These were baked overnight at temperatures no higher than 50-55°C. The first step of the immunoperoxidase procedure involved deparaffinizing the slides with xylene followed by rehydration. The slides were immersed in 2N HCl for one hour and then in 0.1M $Na_2B_4O_7$. The slides were washed in phosphate buffered saline and treated with goat serum. Specimens were incubated with anti-BUdR monoclonal antibody (diluted 1:40) for one hour, washed and treated with goat anti-mouse antibody. Mouse IgG peroxidase was then added, followed by staining with 3,3'-diaminobenzidine tetrahydrochloride (DAB). Cells that contained BUdR were identified by the presence of a brownish pigment over nuclei. (See Appendix B for details of procedure).

3. Mitotic figures

Assessment of mitotic figures was carried out by using metaphase arrest technique. Animals received a s.c. injection of colchicine (1 mg/kg b.w.) two and a half hours prior to termination. The colon was removed and fixed in formalin for at least 24 hours. Sections were prepared and stained as outlined above in H-2 (crypt height). Metaphase figures were identified by their dark blue coloration and specific chromosomal arrangement around centromeres.

I. Measurement of Serum T_3

Blood was obtained by cardiac puncture. The serum was separated and stored at -80°C. Serum T_3 was measured by radioimmunoassay using the Immuchem ^{125}I Triiodothyronine kit (ICN Biomedicals, Inc.). The assay was carried out by Dr. Bill Woodward's laboratory (Department of Nutritional Sciences, University of Guelph). The principle of radioimmunoassay is based on the ability of an antibody to bind its antigen. The radioactive antigen ($T_3{}^{125}I$) and nonradioactive antigen (T_3) compete for binding sites on its specific antibody. The more nonradioactive antigen present, the less radioactive antigen binds until free and antibody-bound antigen reach equilibrium. In the Immuchem T_3 assay, the antibody is covalently bound to the inner surface of a polypropylene tube. Thus antibody-antigen complex is also bound to the tube wall. Once the assay is completed, the free

antigen is aspirated off and the antibody-bound antigen remains attached to the tube. To determine the level of antibody-bound $T_3{}^{125}I$, the coated tube is counted in a gamma scintillation counter. Levels of T_3 in the serum are then determined graphically from a standard curve.

The first step of the assay procedure involved reconstituting the lyophilized standards by adding 2.0 ml of distilled water to the "0 ng/dL" standard and 1.0 ml distilled water to the remaining standards (25, 50, 100, 200, 400 and 800 ng/dL). These were allowed to sit at room temperature for 15 minutes. Duplicate anti-T_3 tubes were placed in a test tube rack. 100 μl of each standard, control or sample was pipetted into the coated tubes. Then 1.0 ml of $T_3{}^{125}I$ was added to each tube and vortexed briefly. The tubes were incubated for 120 minutes at 37°C. The tubes were then aspirated or decanted in the same order as pipetted and counted in a gamma counter for one minute. The intra and inter assay variation were 1-2 % and 4-6 %, respectively, with a 95-98 % recovery. (See appendix C for details of the calculations).

J. Lipid Analysis

1. Extraction of lipids

At the time of termination, livers were removed from the animals and the colonic mucosa were obtained by scraping the surface of the colon with a glass slide. The livers and

mucosa were immediately frozen at -80°C for future analysis. Frozen tissues were thawed at room temperature and lipids were extracted according to the method of Folch et al. (1957). The tissue samples were homogenized in chloroform/methanol (2:1) containing 0.02% BHT. The mixture was then filtered and the filtrate was rinsed with 0.29% NaCl solution (0.2 x volume). The supernatant was aspirated off and the remaining lipid extract was used for lipid analysis.

2. Separation of lipid fractions by thin layer chromatography

Thin layer chromatography on Silica Gel 60 (Merck) was used to separate the desired fractions from lipid extracts. The location of various lipid classes was determined by spotting standard samples on the plate. The solvent system petroleum ether/ethyl ether/glacial acetic acid (80:21:1) was used to isolate total triglycerides (TG) and total phospholipids (PL). Individual phospholipids, phosphotidylcholine (PC) and phosphotidylethanolamine (PE), were separated with the solvent system chloroform/methanol/distilled water (65:25:4). Following development in the solvent system, the plates were removed, dried and sprayed with dichlorofluorescein to visualize the specific bands under ultraviolet light. Appropriate standards were used to identify the bands (Sigma Chemical Co., St. Louis, MO).

3. Fatty acid analysis

The corresponding TG, PL, PE and PC bands were immediately scraped from the plates into test tubes. An internal standard, heptadecanoic acid (Sigma Chemical Co., St. Louis, MO), was added to the scrapings and these were stored in petroleum ether. Methyl esters were prepared by adding three ml of 0.5N hydrochloric gas in methanol to each lipid sample. The test tubes were immediately capped and left for 20 minutes at room temperature. One ml of distilled water was added to each tube following methylation. The samples were vortexed and then centrifuged for 5 minutes at approximately 1300 rpm in the TJ-6R centrifuge. The layer containing the fatty acid methyl esters (supernatant) was removed and dried under nitrogen. The fatty acid methyl esters were analyzed using a Hewlett-Packard 5890A gas chromatogram on a DB-225 capillary column (30m x 0.25mm). The gas chromatogram was equipped with a flame ionization detector. The injector and detector temperatures were programmed at 205°C, with a helium carrier gas flow rate of 50 ml/min.

4. Quantitation of TG, PC and PE

The phospholipids and triglyceride were quantified by the method of Christie et al. (1970). The mass of the fatty acids present in the sample was calculated by comparison with a known amount of internal standard, heptadecanoic acid (15:0). The average molecular weight of the chain lengths present in

the greatest quantity was then determined. The total weight of PC, PE or TG was calculated based on the observation that the 16, 18, and 20 carbon chains constituted the majority of the fatty acids present. This method of quantitation has been used by others (Aukema et al., 1992; Karmiol and Bettger, 1988).

L. Photography

All pictures were taken using Kodak Panatomic-X black and white film in a Nikon FX-35A camera which was attached to a Nikon microscope.

M. Statistical Analysis

Data analysis was performed by analysis of variance in combination with Duncan's multiple range test using SAS software (SAS User's Guide, 1988). For all tests a probability of less than or equal to 5% ($P \leq 0.05$) was considered significant.

Chapter IV

EFFECT OF T_3 ON THE INDUCTION AND GROWTH OF ACF IN MURINE COLON.

1. Introduction

Epidemiological investigations regarding the role of thyroid hormone in neoplastic events have been conflicting. However, laboratory studies, both *in vitro* and *in vivo*, have implicated thyroid hormone as a risk factor in cancer development (Borek et al., 1985; Borek et al., 1983; Cave et al., 1977; Guersney and Leuthauser, 1987; Kumar et al., 1979; Lopez et al., 1989). Animal studies indicate that thyroid hormone treatment is associated with increased frequencies of some forms of cancer, enhanced growth of transplantable tumours and aggression of metastatic proliferation. In contrast, hypothyroidism appears to reverse some of these effects (Kellen 1972; Kumar et al., 1979). It is generally indicated that thyroid hormones serve as crucial factors in the initiation of cellular neoplastic transformation (Borek et al., 1985; Borek et al., 1983; Lopez et al., 1989).

A systematic evaluation of the possible role of thyroid hormones in modulating carcinogenic responses in the whole organism remains to be undertaken. In the present study, the murine model was used to investigate the effect of T_3 on the induction and growth of ACF - putative preneoplastic lesions of colon cancer. To determine if the time of administration

of the hormone is important in these events, T_3 was added to the diet at the time of initiation in the first study and one week after the last injection in the second study.

2. Materials and Methods

a. Animals:

Female CF1 mice weighing approximately 20 g were used in both studies I and II.

b. Study design:

Study I: The experiment is schematically presented in Figure 9. Fifty-two mice were randomly allocated to five treatment groups with eight animals in each and a control group containing twelve animals. All animals were injected twice with AOM (5 mg/kg i.p.), on days 8 and 12. Control animals were maintained on the control diet (AIN-76) for the duration of the experiment (Group A). Two treatment groups were initially given diets containing either 0.5 or 1.0 ppm T_3 for one week and then switched over to control diets immediately following the first AOM injection (Groups B and C, respectively). The remaining three treatment groups were initially given control diets for one week and then switched over to diets containing either 0.5, 1.0, or 2.0 ppm T_3 immediately following the first AOM injection (Groups D, E and F, respectively). Approximately two hours prior to killing by CO_2 asphyxiation on day 32, the mice were injected with 1 mg/kg colchicine i.p. to assess the proliferative status of

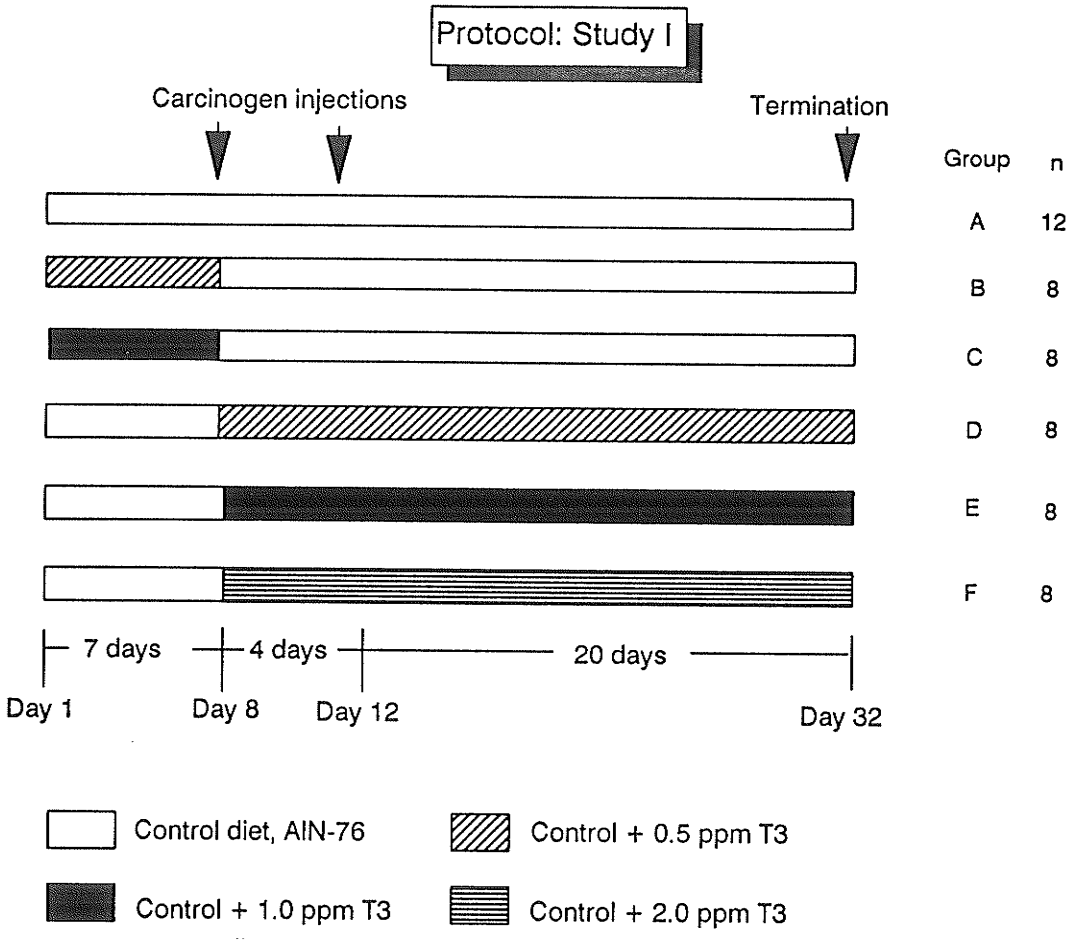


Fig. 9. Schematic representation of the experimental protocol for mice given diets containing T3 prior to and during AOM exposure.

the colon.

Study II: The study design is schematically represented in Figure 10. Forty mice were randomly allocated to five groups with eight in each. Animals received two injections of AOM (5 mg/kg i.p.) one week apart on days 1 and 8. All mice were placed on the control diet, AIN-76, on day 1. One week after the second injection on day 15, they were switched to their test diets. The control group continued to receive the control diet. The other four groups were given diets containing 0.1, 0.25, 0.5 and 1.0 ppm T_3. All animals were killed on day 43.

3. Results

Study I: Table 2 shows initial and weekly mean body weights for animals in each group. In general, body weights remained quite stable throughout the experiment. Animals fed diets supplemented with 2 ppm T_3 (group F) displayed slightly but significantly lower mean body weights initially and again at week 2, but at no other time point.

The average height and mitotic figures of colonic crypts in the rectal and middle portion of the colon is presented in Table 3. All treatment groups demonstrated a significant increase in average crypt height in both the first two and second two centimetres of the colon compared to controls ($P \leq 0.05$). No significant changes in mitotic figures per crypt

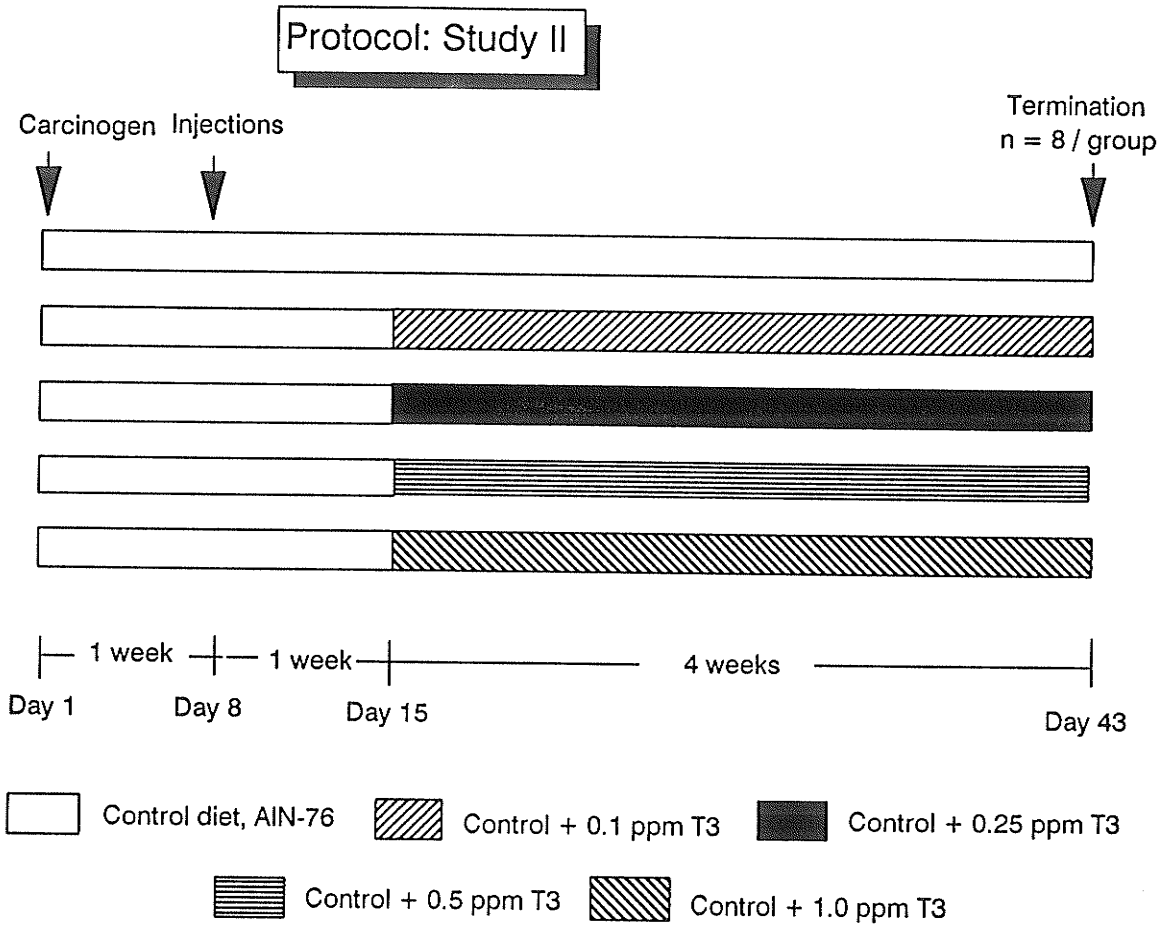


FIG. 10. Schematic representation of experimental protocol for mice given diets containing T3 one week following AOM treatment.

TABLE 2. Body weights of mice fed T_3 in the diet before or during AOM treatment.

Group	Protocol[1]	Body weights (g)				
		Initial	Week 1	Week 2	Week 3	Week 4
A	con-aom-con	20.0 ± 0.2	21.1 ± 0.3	22.8 ± 0.3	23.9 ± 0.4	25.1 ± 0.3
B	0.5-aom-con	19.9 ± 0.2	21.5 ± 0.4	24.3 ± 0.4	25.4 ± 0.4	26.8 ± 0.6
C	1.0-aom-con	20.1 ± 0.2	20.8 ± 0.5	23.6 ± 0.7	25.0 ± 0.8	26.0 ± 0.7
D	con-aom-0.5	20.4 ± 0.2	21.0 ± 0.2	22.6 ± 0.4	24.8 ± 0.3	25.6 ± 0.5
E	con-aom-0.1	19.5 ± 0.2	20.4 ± 0.2	21.6 ± 0.4	24.5 ± 0.5	25.3 ± 0.4
F	con-aom-2.0	18.4 ± 0.5[a]	20.3 ± 0.4	20.4 ± 0.6[a]	24.6 ± 0.5	24.1 ± 0.6

[1]Refer to Fig. 9 for details of protocol.
Values expressed as means ± SEM. [a]Significantly different from control ($P \leq 0.05$).

TABLE 3. Effect of T_3 on average crypt height and number of mitotic figures in colonic crypts of mice.

Group	Protocol[3]	CH[1]		MF[2] per crypt	
		Rectal[4]	Mid[5]	Rectal	Mid
A	con-aom-con	19.6 ± 0.2^{a}	22.1 ± 0.4^{a}	2.8 ± 0.3	2.3 ± 0.3
B	0.5-aom-con	20.9 ± 0.3^{b}	24.2 ± 0.4^{bc}	4.4 ± 0.3	4.2 ± 0.6
C	1.0-aom-con	22.6 ± 0.4^{cd}	23.6 ± 0.4^{b}	3.4 ± 0.7	2.8 ± 0.6
D	con-aom-0.5	21.7 ± 0.4^{bc}	25.3 ± 0.4^{cd}	3.6 ± 0.8	4.2 ± 0.9
E	con-aom-1.0	23.5 ± 0.6^{d}	25.7 ± 0.6^{d}	4.1 ± 1.0	4.3 ± 1.5
F	con-aom-2.0	24.8 ± 0.3^{e}	26.3 ± 0.6^{d}	2.6 ± 0.4	3.6 ± 2.2

[1]Crypt height.
[2]Mitotic figures.
[3]Refer to Fig. 9 for details of protocol.
[4]cm 1-2 from the rectal end of the colon.
[5]cm 3-4 form the rectal end of the colon.
Values expressed as means ± SEM. Means not sharing a common superscript are significantly different from each other ($P \leq 0.05$).

section were reported for any group. However the values clearly indicate a definite trend toward increased proliferation in colonic crypt cells of animals fed diets containing T_3, irrespective of dietary protocol, compared to control animals.

Histological sections of colons were assessed for proliferative indices as well as any morphological alterations. It was noted that the colonic crypts of T_3 treated animals were elongated and exhibited cellular crowding and altered nuclear arrangement. These effects were most prominent in the highest dose of T_3 treatment (Figure 11).

The total number of ACF per colon and the distribution of ACF according to crypt multiplicity is shown in Table 4. All treatment groups displayed an increase in mean number of ACF per colon compared to control animals. Significant increases ($P \leq 0.05$) were observed in animals fed either 1.0 or 2.0 ppm T_3 (Groups E and F) during and after initiation with AOM compared to the control group (12.4 and 19.8 vs 4.6, respectively). Differences in mean number of ACF between animals fed T_3 diets pre-initiation (Groups B and C) and control animals (Group A) were not significant.

Animals fed T_3 diets during and post-initiation displayed a significant increase in mean number of ACF per colon

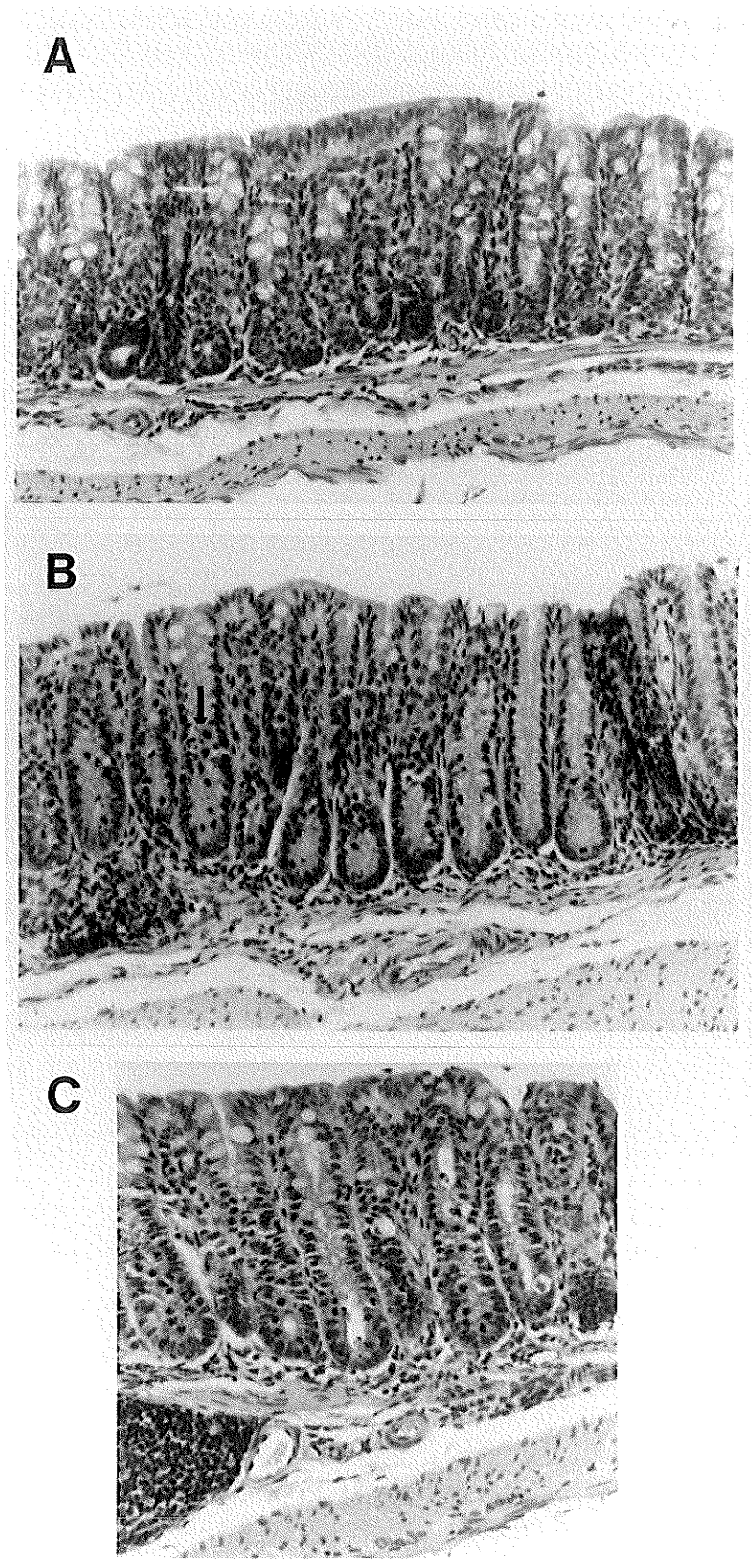

A
B
C

TABLE 4. Effect of T_3 on crypt multiplicity of ACF and total ACF per colon in mice given T_3 in the diet pre- or during initiation.

Group	Protocol[2]	CM[1] AC 1	CM[1] AC 2	CM[1] AC 3-4	Total ACF per colon
A	con-aom-con	4.2 ± 0.6	0.4 ± 0.2	0	4.6 ± 0.7
B	0.5-aom-con	7.6 ± 2.0	1.1 ± 0.5	0.1 ± 0.1	8.9 ± 2.5
C	1.0-aom-con	5.0 ± 0.5	1.0 ± 0.4	0	6.0 ± 0.6
D	con-aom-0.5	8.1 ± 2.2	2.3 ± 0.3[a]	0.3 ± 0.2	10.6 ± 2.4
E	con-aom-1.0	11.0 ± 1.4[a]	1.4 ± 0.4	0	12.4 ± 1.6[a]
F	con-aom-2.0	15.1 ± 4.1[a]	4.0 ± 1.0[a]	0.6 ± 0.3[a]	19.8 ± 5.0[a]

[1]Crypt multiplicity or number of crypts per focus: AC 1 = 1 crypt per focus; AC 2 = 2 crypts per focus; AC 3-4 = 3 to 4 crypts per focus.
[2]Refer to Fig. 9 for details of protocol.
Values expressed as means ± SEM. [a]Significantly different form control ($P \leq 0.05$).

containing either 1, 2 or 3-4 crypts per focus when compared to the control group. Increases in the animals fed diets containing T_3 prior to initiation with AOM did not attain statistical significance.

Study II: The mean number of ACF per colon and crypt multiplicity are shown in Table 5. The animals eating diets containing T_3 had more ACF than control animals but this was significant only for 0.5 ppm T_3. The mean number of ACF were lower in this study as compared to the data in study I.

4. Discussion

This study was conducted to explore whether T_3 affects the proliferative status of the colonic epithelium, as well as the induction and crypt multiplicity of ACF, reported to be putative preneoplastic lesions in rodent colons (Bird, 1987; McLellan and Bird, 1991, McLellan et al., 1991a). The findings of the present study demonstrate that short term chronic feeding of a diet with T_3 results in hyperplasia of the colonic epithelial cells and an increase in the development and growth of ACF in the mouse colon.

T_3 was administered to the mice through diet, a route commonly used in animal studies to increase the biologically active T_3 levels in the blood (Perry et al., 1988; Woods and Woodward, 1991). It was noted that body weights remained quite stable

TABLE 5. Effect of T_3 on crypt multiplicity of ACF and total ACF per colon in mice given T_3 in the diet one week after AOM exposure.

Group	CM[1] AC 1	AC 2	AC 3-4	Total ACF per colon
Control	2.6 ± 0.8	0.4 ± 0.3	0	3.0 ± 0.8
0.1 ppm T_3	3.6 ± 0.5	1.5 ± 0.4	0	5.1 ± 0.7
0.25 ppm T_3	3.6 ± 0.8	1.9 ± 1.0	0.1 ± 0.1	5.6 ± 1.6
0.5 ppm T_3	5.5 ± 0.9[a]	0.8 ± 0.3	0	6.3 ± 0.9[a]
1.0 ppm T_3	3.9 ± 0.4	0.1 ± 0.1	0.1 ± 0.1	4.1 ± 0.4

[1]Refer to Table 4.
Values expressed as means ± SEM. [a]Significantly different from control ($P \leq 0.05$).

in all groups throughout the experiment indicating that T_3 was well tolerated by the animals and was non-toxic.

In study I, both the mean number of ACF and crypt multiplicity were increased with T_3 addition to the diet. However, significant differences in these parameters were observed only in animals switched to T_3 diets at the time of AOM treatment suggesting that T_3 cooperated in the events associated with induction and growth of ACF. In addition, study II showed that the effect of feeding T_3 one week after initiation was not as pronounced as when T_3 was given simultaneously with AOM. These observations are consistent with the findings of other investigators who demonstrated that exposure to thyroid hormone at the time of initiation enhanced cellular transformation *in vitro*. It has been shown that various cell cultures are more vulnerable to transformation by activated Ha-ras oncogenes, x-irradiation, viruses or chemicals in media supplemented with T_3 as compared to T_3-depleted media (Borek et al., 1985; Guersney and Leuthauser, 1987; Lopez et al., 1989). Furthermore, Lopez and coworkers (1989) found that culture media supplemented with T_3 12 hour prior to benzo-a-pyrene (B[a]P) exposure and then removed for 48 hours had less transformed cells then culture media supplemented with T_3 at the time of B[a]P exposure and maintained 48 hours thereafter. These results suggest that T_3 plays a critical role in the initiation of neoplastic transformation and that the timing of

T_3 exposure is a critical factor when studying neoplastic events.

Whether T_3 affects the induction of transformed cells or affects other stages of carcinogenesis such as promotion or progression has not been established. Studies conducted in animal models have shown that thyroid hormone treatment enhances the growth and metastasis of implanted cancer cells in the murine system (Kumar et al., 1979) suggesting a possible role for thyroid hormones during the later stages of carcinogenesis. It was demonstrated in the second study that the effect of T_3 is not as pronounced when introduced in the diet one week after initiation, the protocol commonly used to study modulation of promotion in carcinogenesis.

In the first study, proliferative status of colonic epithelium was quantified since increased cell proliferation has been associated with increased risk for developing colon cancer (Jacobs, 1988). The average crypt height was greater for all treatment groups regardless of T_3 feeding schedule. This was accompanied by increased number of mitotic figures indicating a trend toward accelerated colonic cell production in the crypts and unbalanced cell growth. It is interesting to note that although colonic hyperplasia was observed in all groups given T_3 diets, the induction and growth of ACF were increased only in those groups which received T_3 diets during as well as

after carcinogen treatment. This finding suggests that hyperplastic responses induced by T_3 treatment may not be the main causative event associated with increased growth of ACF. This raises some interesting questions regarding the mode of action of thyroid hormones in regulating colonic cell growth and in altering the responses of colonic cells to a colon carcinogen.

To the authors' knowledge, this investigation is the first to examine the effect of T_3 in the early stages of carcinogenesis in the whole organism. The findings of the present study demonstrated that CF1 mice tolerate T_3 in the diet up to a dose level of 2.0 ppm and suggest that the murine model can be used to investigate the effect of thyroid hormone on the carcinogenic events of the colon.

Chapter V

EFFECT OF T_3 ON THE INDUCTION AND GROWTH OF ACF IN RAT COLON

1. Introduction

It is well known that thyroid hormones exert a variety of physiological responses in a species-specific and organ-specific manner. Although colon cancer can be induced in a wide variety of animal models, rats are a commonly employed species in the study of experimental colon carcinogenesis. The advantage of the rat model is that a single injection of a colon specific carcinogen will induce tumours in 20 - 30 weeks. In mice however, multiple injections of low dosages of the carcinogen are required to induce tumours, due to their lower tolerance levels. Multiple injection protocols do not allow one to measure or assess the cancer modulating effect of an agent in a stepwise manner. Therefore, it was of interest to investigate the responses of rat colonic tissue to elevated levels of T_3 with respect to the induction and growth of ACF and proliferative status of the colon.

2. Materials and Methods

a. Animals:

Sprague-Dawley rats weighing approximately 130 and 105 g were used in studies I and II, respectively.

b. Study Design:

Study I: The study design is schematically represented in Figure 12. Sixty rats were injected with 10 mg/kg AOM s.c. and immediately placed on the control diet. One week later, animals were randomly allocated to four groups of 15 each. One group continued to receive the control diet; the other three were given diets containing either 0.1, 0.25 or 0.5 ppm T_3. Animals were killed at two different time points. Eight animals per group were killed on day 26, three days earlier than originally scheduled, due to the toxicity of 0.5 ppm T_3. At this time, the remaining seven rats eating diets containing 0.5 ppm T_3 were switched back to the control diet for the remainder of the experiment. All remaining animals were killed three weeks later on day 47. Four rats per group were injected with colchicine and three with bromo-deoxyuridine prior to killing. Blood was withdrawn from each animal by heart puncture. The serum was removed and stored at -80°C for future analysis of T_3 levels.

Study II: The study design is schematically represented in Figure 13. Twenty-four rats were randomly allocated to four groups with six animals in each. All animals were injected twice with AOM (5 mg/kg s.c.), on days 1 and 5. Animals were placed on diets immediately following the first injection. One group received the control diet (AIN-76). The other three groups were given diets containing 0.05, 0.1 and 0.25 ppm T_3. All animals were killed on day 29. At the time of death, the

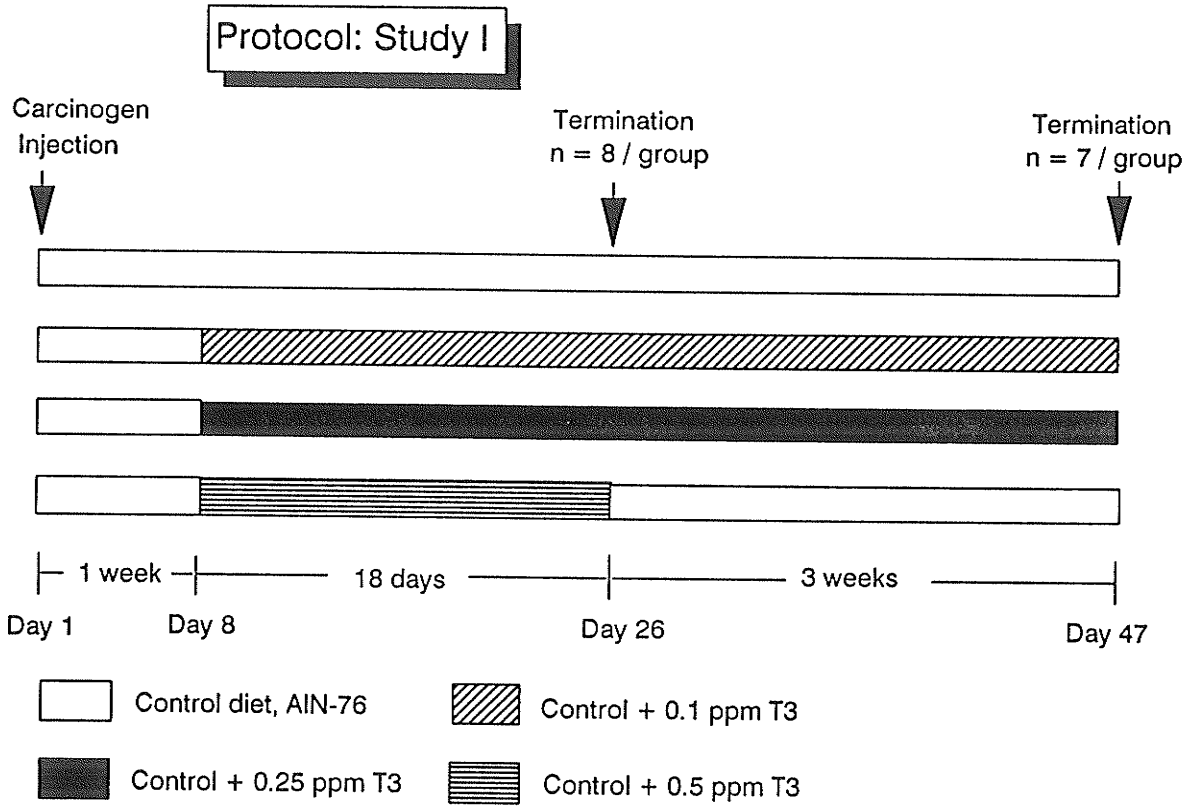


FIG. 12. Schematic representation of the experimental protocol for rats given diets containing T3 one week following AOM exposure.

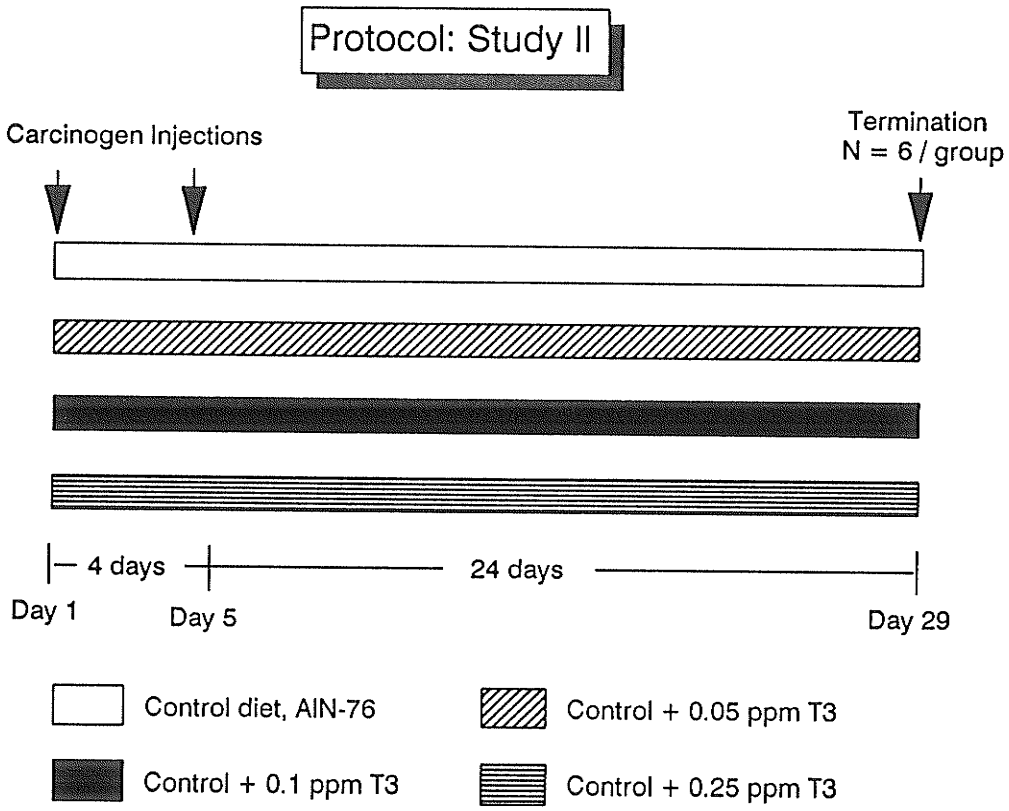


FIG. 13. Schematic representation of the feeding schedules for rats given diets containing T3 at the time of AOM treatment.

weight and length of the colon were measured for each animal. Also, the liver, kidney and heart were removed and the weights of these organs were recorded.

3. Results

Study I: The mean body weights of the animals are presented in Table 6. Body weights were not affected by T_3 feeding. Animals on 0.1 ppm T_3 had higher weights than all other groups throughout the experiment but this was only significant for week two. It is worth emphasizing that 0.5 ppm T_3 induced toxic responses in the rats which included hyperventilation and immobility. Therefore, the group receiving this dose level in the diet was given the control diet after 18 days of T_3 feeding.

The food consumption data is provided in Table 7. Rats receiving T_3 diets ate significantly more than control animals at all time points recorded ($P \leq 0.05$). The amount of food consumed by animals on 0.5 ppm T_3 gradually decreased after being switched back to control diets but still remained significantly above control levels.

Tables 8, 9 and 10 outline the data for mean number of ACF per colon, distribution of crypt multiplicity, mean multiplicity and mean size of ACF. There were no significant differences in any of these parameters for animals eating diets containing

TABLE 6. Body weights of rats given T_3 in the diet one week after AOM exposure.

Group	Body weight (g)					
	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
Control	125 ± 6	167 ± 5	190 ± 5	215 ± 12	235 ± 12	252 ± 12
0.1 ppm T_3	135 ± 6	179 ± 5	212 ± 7[a]	226 ± 5	246 ± 5	259 ± 5
0.25 ppm T_3	134 ± 5	170 ± 4	195 ± 4	219 ± 7	237 ± 8	247 ± 9
0.5 ppm T_3[1]	134 ± 4	169 ± 2	190 ± 3	218 ± 6	243 ± 7	257 ± 8

[1]Animals receiving 0.5 ppm T_3 in the diet were placed on the control diet on day 26 for the remainder of the experiment.
Values expressed as means ± SEM. [a]Significantly different from control ($P \leq 0.05$).

TABLE 7. Effect of T_3 on daily food consumption of rats injected with AOM.

Group	Food Consumption (g/day)				
	Week 2	Week 3	Week 4	Week 5	Week 6
Control	16.0 ± 0.6^{a}	18.0 ± 0.7^{a}	18.4 ± 1.3^{a}	17.2 ± 1.7^{a}	18.5 ± 1.2^{a}
0.1 ppm T_3	18.0 ± 0.1^{b}	20.6 ± 0.6^{b}	20.9 ± 0.3^{ab}	21.7 ± 0.4^{b}	21.6 ± 0.2^{b}
0.25 ppm T_3	17.4 ± 0.6^{ab}	21.5 ± 0.4^{b}	25.2 ± 1.8^{b}	26.1 ± 1.3^{c}	25.3 ± 0.5^{c}
0.5 ppm T_3[1]	18.3 ± 0.4^{b}	23.9 ± 1.0^{c}	29.8 ± 1.6^{c}	23.4 ± 0.5^{bc}	21.4 ± 0.5^{b}

[1]Animals receiving 0.5 ppm T_3 in the diet were placed on the control diet on day 26 for the remainder of the experiment.
Values expressed as means ± SEM. Means not sharing a common superscript are significantly different from each other ($P \leq 0.05$).

TABLE 8. Effect of administering T_3 one week after AOM exposure on total ACF per colon and crypt multiplicity of ACF in rats terminated on day 26.

Group	Total ACF per colon	CM[1]		
		AC 1	AC 2	AC 3+
Control	154.0 ± 46.0	98.5 ± 24.0	47.5 ± 19.8	8.0 ± 3.1
0.1 ppm T_3	155.0 ± 25.5	97.5 ± 18.2	49.3 ± 5.5	8.3 ± 3.3
0.25 ppm T_3	124.0 ± 18.1	95.5 ± 10.7	27.3 ± 6.9	1.3 ± 0.6
0.5 ppm T_3	104.5 ± 16.4	81.8 ± 15.4	21.0 ± 3.4	1.8 ± 1.1

[1]Crypt multiplicity, number of crypts per focus: AC 1 = 1 crypt per focus; AC 2 = 2 crypts per focus; AC 3+ = 3 to 5 crypts per focus. Values expressed as means ± SEM.

TABLE 9. Effect of administering T_3 in the diet one week after AOM exposure on total ACF per colon and crypt multiplicity of ACF in rats terminated on day 47.

Group	Total ACF/colon	CM[1]				
		AC 1	AC 2	AC 3	AC 4	AC 5+
Control	137 ± 23	58.6 ± 12.3	50.7 ± 10.8	19.3 ± 4.9	5.7 ± 2.4	2.7 ± 1.4
0.1 ppm T_3	92 ± 10	38.4 ± 4.7	30.4 ± 3.2	12.9 ± 3.5	6.1 ± 1.9	4.0 ± 1.5
0.25 ppm T_3	111 ± 12	50.0 ± 8.5	37.9 ± 4.0	14.1 ± 1.9	5.7 ± 2.0	2.9 ± 1.5
0.5 ppm T_3[2]	109 ± 14	34.4 ± 7.4	42.6 ± 5.6	18.6 ± 3.1	8.3 ± 2.0	4.7 ± 1.8

[1]Crypt multiplicity, number of crypts per focus: AC 1 = 1 crypt per focus; AC 2 = 2 crypts per focus, etc; AC 5+ = 5 to 8 crypts per focus.
[2]Animals receiving 0.5 ppm T_3 in the diet were placed on control diet on day 26 for the remainder of the study.
Values expressed as means ± SEM.

TABLE 10. Effect of T_3 on mean multiplicity and mean size of ACF in rat colon.

	Termination 1		Termination 2	
Group	Mean multiplicity[1]	Mean size[2]	Mean multiplicity	Mean size
Control	1.38 ± 0.05	3.28 ± 0.20	1.90 ± 0.19	3.69 ± 0.43
0.1 ppm T_3	1.43 ± 0.03	3.70 ± 0.09	1.97 ± 0.16	4.33 ± 0.35
0.25 ppm T_3	1.23 ± 0.03	3.38 ± 0.08	1.90 ± 0.14	3.91 ± 0.29
0.5 ppm T_3[3]	1.25 ± 0.07	3.43 ± 0.30	2.20 ± 0.16	4.11 ± 0.28

[1]Mean multiplicity refers to the average no. of aberrant crypts per focus.
[2]Mean size refers to the average size of each focus.
[3]Animals receiving 0.5 ppm T_3 in the diet were placed on control diet after termination 1 for the remainder of the study.
Values expressed as means ± SEM.

T_3 compared to controls on either day 26 or day 47.

The proliferative features of the colon are presented in Table 11. The mean number of mitotic figures in the rectal and mid colon was not significantly different for animals given T_3 in the diet compared to controls. T_3 feeding did however result in a significant increase in mean number of labelled cells in the rectal colon of rats given 0.1 or 0.25 ppm T_3 in the diet compared to controls ($P \leq 0.05$). No significant differences in number of labelled cells were found in the mid colon.

Table 12 shows that the average height of colonic crypts was significantly increased T_3 (33.5 ± 0.5, 34.1 ± 0.4 and 35.9 ± 0.4 for 0.1, 0.25 and 0.5 ppm T_3 respectively vs 29.2 ± 0.4 cells for control animals). Administration of 0.1 and 0.25 ppm T_3 in the diet increased total plasma T_3 concentrations. This increase was significant for 0.25 ppm T_3. The plasma T_3 levels for animals switched from 0.5 ppm T_3 to control diet on day 26 returned back to control levels by the time of the second termination on day 47.

Study II: The body weights of the animals remained quite stable throughout the experiment (Table 13). Control animals had slightly greater body weights than rats given T_3 at week four but this was only significant compared to animals receiving 0.1 ppm T_3.

TABLE 11. Effect of T_3 on the number of mitotic figures and labelled cells in colonic crypts of rats.

	MF[1]		LC[2]	
Group	Rectal colon[3]	Mid colon[4]	Rectal colon	Mid colon
Control	1.9 ± 0.3	2.4 ± 0.2	1.0 ± 0.2	2.9 ± 0.3
0.1 ppm T_3	2.6 ± 0.3	3.0 ± 0.3	2.5 ± 0.3[a]	2.5 ± 0.3
0.25 ppm T_3	1.4 ± 0.3	2.8 ± 0.3	2.3 ± 0.3[a]	3.0 ± 0.3

[1]Mitotic figures.
[2]Bromo-deoxyuridine labelled cells.
[3]cm 1 from the rectal end of the colon.
[4]cm 6 from the rectal end of the colon.
Values expressed as means ± SEM. [a]Significantly different from control ($P \leq 0.05$).

TABLE 12. Effect of T_3 on the average height of colonic crypts and serum T_3 concentrations in rats.

Group	CH[1]	Serum T_3 (ng/dL)
Control	29.2 ± 0.4	126 ± 12
0.1 ppm T_3	33.5 ± 0.5[a]	151 ± 12
0.25 ppm T_3	34.1 ± 0.4[a]	200 ± 30[a]
0.5 ppm T_3[1]	35.9 ± 0.4[a]	119 ± 6

[1]Animals receiving 0.5 ppm T_3 in the diet were placed on control diet after termination 1 for the remainder of the study.
Values expressed as means ± SEM. [a]significantly different from control.

TABLE 13. Body weights of rats administered T_3 in the diet at the time of AOM exposure.

	Body Weights (g)				
Group	Initial	Week 1	Week 2	Week 3	Week 4
Control	101 ± 3	129 ± 4	156 ± 4	183 ± 4	205 ± 5
0.05 ppm T_3	101 ± 3	120 ± 4	155 ± 3	177 ± 5	196 ± 4
0.1 ppm T_3	103 ± 3	124 ± 3	144 ± 6	172 ± 5	188 ± 6[a]
0.25 ppm T_3	110 ± 6	126 ± 6	156 ± 5	180 ± 6	190 ± 6

Values expressed as means ± SEM. [a]Significantly different from control.

The organ weights and colon lengths are presented in Table 14. There was a considerable amount of variation in these measurements. However, the most consistent finding was that animals eating diets containing 0.25 ppm T_3 had significantly higher weights for the liver, kidney and heart. The weight of the colon was significantly greater for control animals compared to those receiving 0.1 ppm T_3. There was no difference in the length of the colon for any group. The colon index was calculated by dividing the colon weight (g) by the colon length (cm) and multiplying by 100. The index was highest for control animals, and this was significant compared to animals receiving 0.1 and 0.25 ppm T_3.

Table 15 shows the data for mean number of ACF per colon and distribution according to crypt multiplicity. T_3 did not affect the distribution profile of ACF at any dose level. There was a great deal of variation in the number of ACF in each group as indicated by the SEM.

4. Discussion

Previously, it was observed that short term chronic feeding of a diet containing T_3 results in hyperplasia of the colonic epithelial cells and enhanced development of ACF in the murine colon. This study was conducted to determine whether T_3 would exert a similar effect in the rat colon. Contrary, to the findings in the murine colon, T_3 did not increase the number

TABLE 14. Effect of T_3 on the weight of various organs and the length of the colon in rats injected with AOM.

	Weights (g)					
Group	Liver	Kidney	Heart	Colon	Colon length (cm)	Colon index
Control	8.7 ± 0.4	0.91 ± 0.05	0.80 ± 0.04	1.24 ± 0.08	16.8 ± 0.6	7.4 ± 0.3
0.05 ppm T_3	8.2 ± 0.2	0.85 ± 0.03	0.82 ± 0.13	1.11 ± 0.04	16.4 ± 0.5	7.8 ± 0.3
0.1 ppm T_3	8.3 ± 0.5	1.02 ± 0.06	0.82 ± 0.04	1.03 ± 0.04[a]	16.2 ± 0.5	6.4 ± 0.2[a]
0.25 ppm T_3	9.7 ± 0.4[a]	1.22 ± 0.05[a]	1.01 ± 0.02[a]	1.10 ± 0.05	17.1 ± 0.8	6.5 ± 0.2[a]

[1](Weight of colon/length of colon) * 100.
Values expressed as means ± SEM. [a]Significantly different from control ($P \leq 0.05$).

TABLE 15. Effect of administering T_3 in the diet one week after AOM exposure on the total ACF per colon and crypt multiplicity of ACF in rats.

Group	Total ACF per colon	CM[1]		
		AC 1	AC 2	AC 3-4
Control	21.2 ± 4.0	15.6 ± 3.0	5.2 ± 1.6	0.4 ± 0.4
0.05 ppm T_3	25.2 ± 7.8	19.5 ± 5.4	5.2 ± 2.5	0.5 ± 0.2
0.1 ppm T_3	24.4 ± 5.2	16.8 ± 3.8	7.2 ± 2.2	0.4 ± 0.2
0.25 ppm T_3	23.5 ± 5.8	15.3 ± 4.0	6.6 ± 1.9	0.3 ± 0.1

[1]Crypt multiplicity, number of crypts per focus: AC 1 = 1 crypt per focus; AC 2 = 2 crypts per focus; AC 3-4 = 3 to 4 crypts per focus.
Values expressed as means ± SEM.

and growth of ACF at any dose level.

In both studies, body weights remained stable for all groups throughout the experiments. T_3 was well tolerated at a level of 0.05, 0.1 and 0.25 ppm. However, it was evident that 0.5 ppm T_3 was highly toxic to the rats, a level which was non-toxic to the murine model. One cannot exclude the possibility that rats are able to absorb more T_3 in the gut than mice and that this may account for differences in tolerance to T_3.

Food consumption was assessed in study I. The amount of food consumed by the rats eating T_3 diets was significantly greater than that for controls at all time points. Given that plasma T_3 concentrations were elevated by T_3 feeding, and that this is associated with an increase in metabolic rate (Acheson et al., 1984), it follows that the animals were eating more to counteract the increase in energy expenditure.

The induction and growth of ACF in the rat colon was not influenced by the addition of T_3 to the diet. In study I, T_3 was introduced in the diet one week after initiation with AOM, the protocol routinely used to examine the effect on promotion. No differences in total number of ACF, crypt multiplicity, mean multiplicity or mean size were observed between the control and treatment groups. Study II examined whether T_3 would affect the development of ACF in the rat

colon if administered to the animals during the time of initiation with AOM and continued throughout the experiment, thereby employing the conditions used in the previous study using CF1 mice. As previously described in chapter four, T_3 had the most pronounced effect on the number and growth of ACF in the murine colon when this protocol was used. However, the results of study II showed that T_3 did not exert a similar effect in the rat model even following this protocol.

In study I, the number of mitotic figures and labelled cells were determined for animals given 0.1 and 0.25 ppm T_3 in the diet. These two measurements are commonly used interchangeably as indicators of cellular proliferation. In this study, the number of labelled cells in the rectal region of the colon of animals receiving 0.1 and 0.25 ppm T_3 was significantly greater than for controls, indicating increased cell proliferation. However, the number of mitotic figures in the rectal region of the colon of treatment groups were not significantly different compared to controls. The fact that the number of mitotic figures do not correspond to the number of labelled cells suggests that these two parameters should not be used interchangeably as risk markers and support the conjecture that usage of various risk markers may not be a reliable measure of disease development (Bird et al., 1987).

The effect of feeding T_3 on the weights of various organs was

assessed in study II. It has generally been indicated that T_3 has an anabolic and hypertrophic effect at physiological levels, leading to an increase in total body weight as well as growth of most tissues. This anabolic effect is due to a general increase in protein and RNA synthesis (Johnson, 1987). In the present study, exogenous T_3 at a level of 0.25 ppm significantly increased the weights of the liver, kidney and heart. In contrast, the weight of the colon and the colon index was highest for control animals, while the length of the colon was similar for all groups. This observation is interesting and does not support the hyperplastic responses seen in the colon. Although the accuracy of the colonic weight and length could respectively be affected by extra saline which was not flushed out upon cleaning and stretching of the colon during cleaning or due to the presence of feces, the reported trend was consistent for the T_3 treated groups. The possibility exists that T_3 treatment may have reduced the moisture content of the tissue resulting in the lower weight.

The present investigations did not demonstrate an effect of T_3 on the development and growth of ACF in rat colon, contrary to the previous findings in the murine colon where T_3 increased the total number and crypt multiplicity of ACF.

The differing effects of T_3 in the colons of CF1 mice and Sprague-Dawley rats may be due to specificity of thyroid

hormone action at its target sites. This is represented by a species and tissue specific effect. For example the activity of L-α-glycerophosphate dehydrogenase (GPD) is influenced by thyroid hormone in various organs of the rat such as liver, kidney, heart, diaphragm, skeletal muscle and adipose tissue. However, in other organs, including the small intestine, GPD is not responsive to thyroid hormone (Lee and Lardy, 1964). Furthermore, although this enzyme is an excellent index of thyroid hormone action in the rat, hepatic GPD fails to respond to T_3 in human and guinea pig liver (Oppenheimer, 1979).

It is important to take into account the fact that there may be differences in the sensitivity of various biological systems to T_4 and T_3 within the same animal. Larsen and Frumes (1977) have demonstrated a direct effect of T_3 on the activity of hepatic mitochondrial GPD in Sprague-Dawley rats, while T_4 had a significant effect on the stimulation of weight gain in the same animals. It is possible that the colon of Sprague-Dawley rats is more sensitive to the biological effects of T_4 rather than T_3. Indeed, it has been reported that T_4 enhances the development of colon tumours in Wistar rats given multiple injections of AOM (Iishi et al., 1992). The study by Iishi et al, (1992) raises the question regarding the biological effect of T_3 in the rat vs the murine system and suggests that T_3 and T_4 must be examined as individual hormones in modulating

cancer processes.

The fact that T_3 exerted different effects on the number and growth of ACF in rat colons compared to the previous findings in murine colons, suggests that these two species may be useful in understanding the multitude of physiological responses induced by thyroid hormones.

Chapter VI

EFFECT OF T_3 ON LIVER AND COLONIC MUCOSA LIPID COMPOSITION IN RATS

1. Introduction

Thyroid hormones affect reactions in almost all pathways of lipid metabolism (Hoch, 1988). Plasma and cell lipid composition are thus also influenced by thyroid status. It has been reported that circulating thyroid hormone levels in blood regulate cholesterol distribution and membrane lipid composition in various tissues (Ruggiero et al., 1990; Ruggiero et al., 1984). Other studies have indicated that enzymes which are responsive to thyroid hormone stimulation are generally those associated with membranes (Davis et al., 1989; Ismail-Beigi, 1992). Therefore, alterations in membrane structure could be the manner in which the activities of many membrane-associated enzymes are altered by thyroid hormones (Ruggiero et al., 1984). Altered membrane lipid composition and activities of membrane associated enzymes may have important implications to the carcinogenic process.

Previous investigations of thyroid hormone induced changes in lipid composition have generally relied on multiple injections of T_3 or T_4 to induce hyperthyroidism (Morini et al., 1991; Raederstorff et al., 1991). In the present studies the effect of continuously administered T_3 on the lipid profile of the liver and colonic mucosa of rats was investigated.

2. Materials and Methods

a. Animals:

Sprague-Dawley rats weighing approximately 210 and 110 g were used for studies I and II, respectively.

b. Study Design:

Study I: The study design is schematically represented in Figure 14. Twenty rats were randomly allocated to four groups of six each. One group received the control diet, AIN-76. The other three groups were placed on diets containing 0.1, 0.25 or 0.5 ppm T_3. All animals were killed three weeks later. At the time of death, the liver was removed from each rat and immediately frozen (-80°C) for future lipid analysis. Blood was withdrawn from each animal by heart puncture. The serum was removed and stored at -80°C for future analysis of cholesterol and T_3 levels.

c. Cholesterol Analysis

Total serum cholesterol was determined using an enzymatic procedure. The cholesterol reagent, standard and control were purchased from Sigma Chemical Co., St. Louis, MO. Twenty μl of distilled water, cholesterol standard and cholesterol control were pipetted into blank tubes, standard tubes and control tubes, respectively. Twenty μl of each sample was pipetted into duplicate sample tubes. One ml of cholesterol reagent was added to each tube and the mixture was vortexed. The tubes were incubated for 15 minutes in a 37°C waterbath. The tubes were then removed from the waterbath; 1.5 ml of

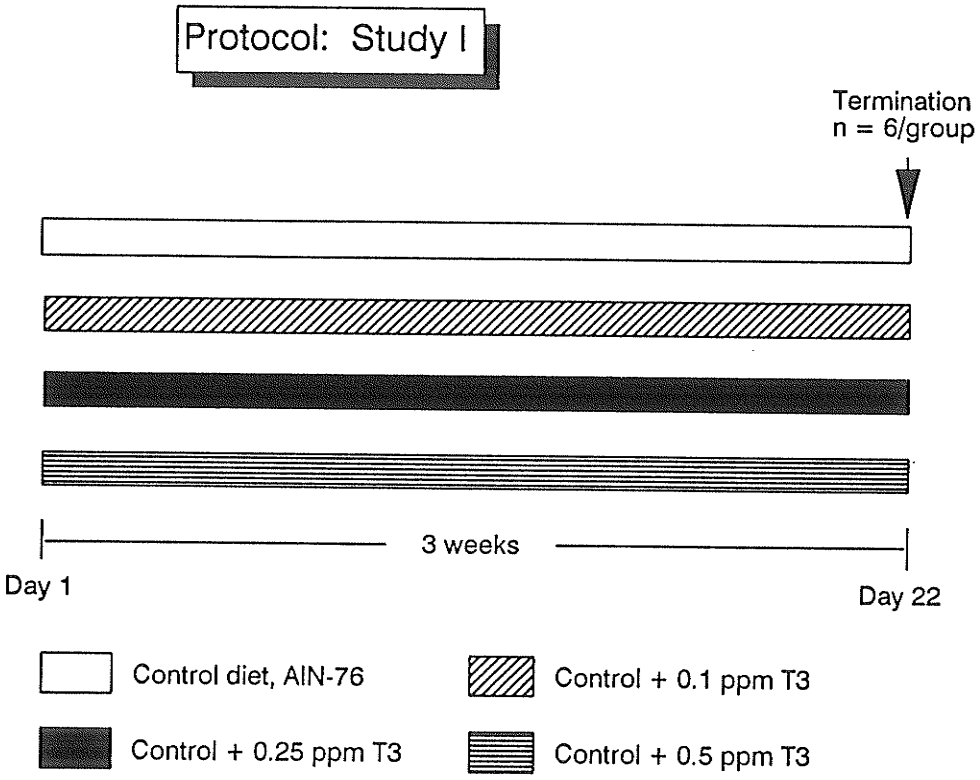


FIG. 14. Schematic representation of the experimental protocol for rats given diets containing T3 at the time of AOM treatment.

water was added to each tube and the mixture was vortexed. The absorbances of the standards, controls, and samples were read in the SP6-300 Spectrophotometer at 505 nm after zeroing the instrument with the blanks. The readings were completed within 20 minutes.

Study II: The study design is schematically represented in Figure 15. Twenty rats were randomly allocated to four groups with six animals in each. One group received the control diet, AIN-76. The other three groups were placed on diets containing 0.05, 0.1 and 0.25 ppm T_3. All animals were killed four weeks later. At the time of death, the weight and length of the colon were measured for each animal. Also, the liver, kidney and heart were removed and the weights of these organs were recorded. The colonic mucosa was obtained from each rat and immediately frozen (-80°C) for future lipid analysis.

3. Results

Study I: Mean body weights of the animals are provided in Table 16. Control animals had slightly greater mean body weights than T_3 treated groups at the start of the experiment; this difference was significant for weeks one, two and three ($P \leq 0.05$).

The food consumption data are presented in Table 17. There were no significant differences in the amount of food consumed at any time point although a trend towards greater consumption

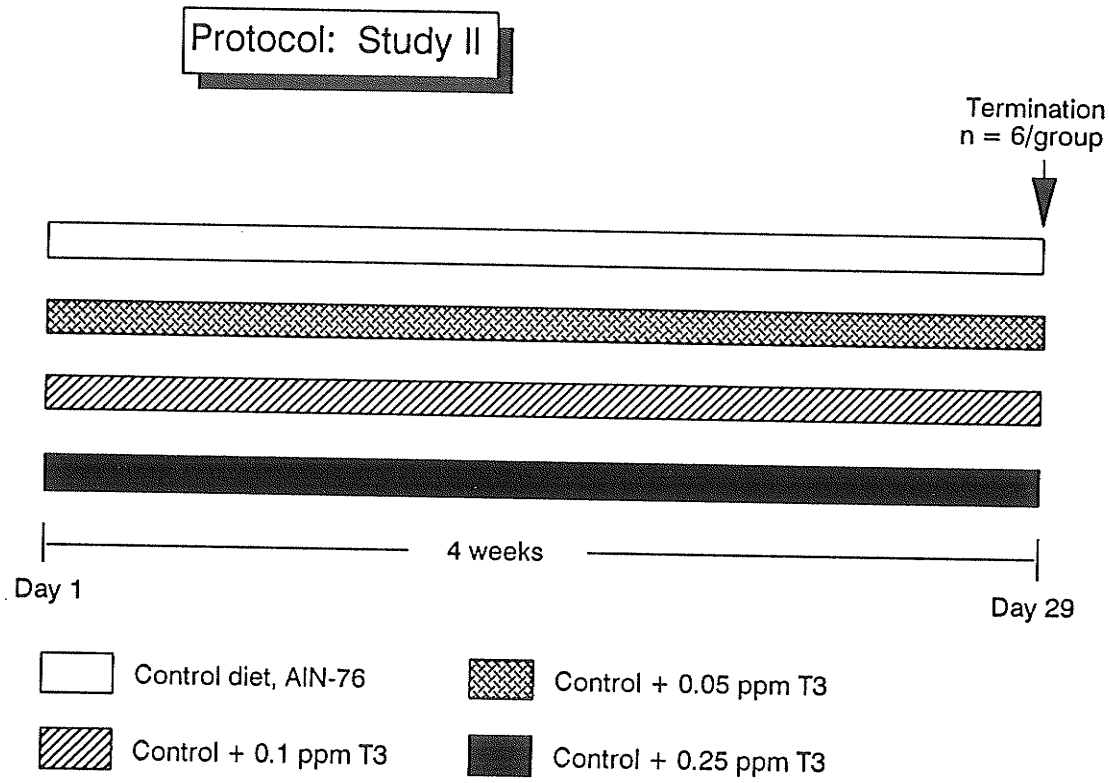


FIG. 15. Schematic representation of the feeding schedules for rat given diets containing T3 for four weeks.

TABLE 16. Body weights of rats given various dosages of T_3 in the diet for three weeks.

Group	Body Weights (g)			
	Initial	Week 1	Week 2	Week 3
Control	221.5 ± 3.8	238.3 ± 8.6	251.5 ± 12.5	259.3 ± 15.7
0.1 ppm T_3	215.2 ± 9.4	215.8 ± 7.4[a]	231.5 ± 7.4	240.7 ± 7.6
0.25 ppm T_3	211.2 ± 1.9	213.3 ± 2.1[a]	223.8 ± 2.0[a]	229.3 ± 2.5[a]
0.5 ppm T_3	205.0 ± 4.7	207.8 ± 3.8[a]	216.8 ± 3.6[a]	222.3 ± 3.4[a]

Values expressed as means ± SEM. [a]Significantly different from control ($P \leq 0.05$).

of T_3 containing diets was evident for weeks two and three.

Table 18 summarizes serum cholesterol levels and total serum T_3 concentrations. Serum cholesterol levels were lower in animals fed T_3 diets compared to the controls. 0.5 ppm T_3 had a significant effect ($P \leq 0.05$). The decrease in serum cholesterol corresponded with an increase in serum T_3 levels.

The effect of T_3 on the total lipid, TG and PL content of the liver is shown in Table 19. The total amount of lipid in the liver was similar in all groups. The TG content was significantly greater for 0.25 ppm T_3 compared to controls. Significantly higher levels of PC and PE were observed in the livers of animals eating diets containing T_3 ($P \leq 0.05$). The relative amounts of PC and PE were altered by 0.1 ppm T_3 in the diet. The ratio of PC:PE was 3.2 ± 0.5 and 1.9 ± 0.1 for 0.1 ppm T_3 and the control, respectively.

Minor changes were detected in the fatty acid composition of liver TG (Table 20). The level of 18:1 tended to be higher in the T_3 treated groups. For animals receiving 0.5 ppm T_3 in the diet, the level of 18:2(n-6) was appreciably lower.

The effect of T_3 on the fatty acid composition of total liver PL is shown in Table 21. The treatment diets significantly lowered the levels of 14:0, 16:0 and 16:1(n-7) and this was

TABLE 17. Effect of T_3 on daily food consumption of rats.

Group	Food Consumption (g/day)		
	Week 1	Week 2	Week 3
Control	17.6 ± 1.9	19.8 ± 2.9	18.5 ± 2.4
0.1 ppm T_3	16.9 ± 0.1	20.4 ± 0.4	20.3 ± 0.9
0.25 ppm T_3	15.2 ± 0.5	21.6 ± 1.8	20.4 ± 1.0
0.5 ppm T_3	16.7 ± 1.0	21.9 ± 0.8	23.1 ± 0.7

Values expressed as means ± SEM.

TABLE 18. Effect of T_3 on serum T_3 and serum cholesterol levels in rats.

Group	Serum T_3 (ng/dL)	Serum cholesterol (mM)
Control	111 ± 10	2.6 ± 0.2
0.1 ppm T_3	134 ± 5	2.4 ± 0.1
0.25 ppm T_3	196 ± 29	2.2 ± 0.2
0.5 ppm T_3	282 ± 53[a]	1.6 ± 0.1[a]

Values expressed as means ± SEM.
[a]Significantly different from control ($P \leq 0.05$).

TABLE 19. Effect of T_3 on the total lipid content, the levels of TG, PC and PE, and relative amounts of PC to PE in rat liver.

Group	Total lipid (mg/g wet wt liver)	Total TG (mg/g wet wt liver)	Total PC (mg/g wet wt liver)	Total PE (mg/g wet wt liver)	PC:PE
Control	50.0 ± 2.3	1.43 ± 0.4[a]	1.04 ± 0.06[a]	0.56 ± 0.05[a]	1.9 ± 0.1[a]
0.1 ppm T_3	49.0 ± 2.7	1.84 ± 0.7[a]	2.13 ± 0.31[c]	0.67 ± 0.14[a]	3.2 ± 0.5[b]
0.25 ppm T_3	48.4 ± 2.1	3.28 ± 1.4[b]	1.64 ± 0.26[b]	0.73 ± 0.07[b]	2.2 ± 0.2[a]
0.5 ppm T_3	52.6 ± 2.5	1.76 ± 0.3[a]	1.48 ± 0.09[b]	0.68 ± 0.08[b]	2.2 ± 0.1[a]

Values expressed as means ± SEM. Means not sharing a common superscript are significantly different from each other ($P \leq 0.05$).

TABLE 20. Effect of T_3 on % fatty acyl composition of TG in rat liver.

Fatty acid	0.0 ppm T_3	0.1 ppm T_3	0.25 ppm T_3	0.5 ppm T_3
14:0	1.13 ± 0.08[a]	0.79 ± 0.06[b]	0.77 ± 0.04[b]	1.11 ± 0.05[a]
16:0	31.41 ± 1.03	29.37 ± 1.76	30.13 ± 1.24	32.83 ± 1.28
16:1(n-7)	4.74 ± 0.35[a]	4.41 ± 0.41[ab]	3.49 ± 0.24[b]	3.80 ± 0.20[ab]
18:0	4.21 ± 0.30[a]	2.51 ± 0.63[b]	3.22 ± 0.08[ab]	4.12 ± 0.19[a]
18:1(n-9)	29.38 ± 0.59[c]	36.37 ± 1.17[a]	34.16 ± 1.72[ab]	32.12 ± 0.57[bc]
18:2(n-6)	20.96 ± 0.65[a]	21.70 ± 3.11[a]	21.45 ± 1.79[a]	14.51 ± 0.16[b]
18:3(n-3)	0.70 ± 0.03[a]	0.27 ± 0.04[b]	0.32 ± 0.05[b]	0.28 ± 0.03[b]
20:0	0.34 ± 0.04	tr	tr	0.28 ± 0.02
20:1	0.23 ± 0.02	0.25 ± 0.03	tr	0.22 ± 0.01
20:3(n-6)	0.25 ± 0.25	0.24 ± 0.09	tr	tr
20:3(n-3)	0.92 ± 0.11	ND	ND	0.79 ± 0.17
20:4(n-6)	3.23 ± 0.25	2.36 ± 0.56	2.73 ± 0.44	3.49 ± 0.37
22:2	ND	ND	1.33 ± 0.61	2.85 ± 0.34
22:3	0.50 ± 0.09[b]	0.23 ± 0.09[b]	0.34 ± 0.09[b]	1.24 ± 0.27[a]
22:4(n-6)	0.44 ± 0.08	0.35 ± 0.13	0.43 ± 0.10	0.56 ± 0.05
22:6(n-3)	0.44 ± 0.08	0.21 ± 0.06	0.36 ± 0.08	0.36 ± 0.08

[1] $\leq$0.2 %.
[2] not detected.
Values expressed as means ± SEM. Differences in horizontal means without a common supercsript are statistically significant ($P \leq 0.05$).

TABLE 21. Effect of T_3 on % fatty acyl composition of PL in rat liver.

Fatty acid	0.0 ppm T_3	0.1 ppm T_3	0.25 ppm T_3	0.5 ppm T_3
14:0	0.32 ± 0.02^{a}	0.16 ± 0.01^{b}	0.07 ± 0.05^{c}	0.21 ± 0.01^{b}
16:0	17:21 ± 0.42^{a}	13.92 ± 0.27^{c}	15.50 ± 0.87^{bc}	16.24 ± 0.39^{ab}
16:1(n-7)	0.94 ± 0.16^{a}	0.52 ± 0.04^{b}	0.42 ± 0.04^{b}	0.58 ± 0.03^{b}
18:0	37.49 ± 0.64	36.12 ± 0.38	36.85 ± 0.91	38.20 ± 0.65
18:1(n-9)	5.49 ± 0.26	5.38 ± 0.14	5.25 ± 0.25	5.54 ± 0.15
18:2(n-6)	9.48 ± 0.24^{a}	8.47 ± 0.37^{b}	8.94 ± 0.24^{ab}	8.77 ± 0.16^{ab}
18:3(n-3)	0.26 ± 0.04	tr[1]	ND[2]	tr
20:2	0.32 ± 0.04	0.31 ± 0.01	0.22 ± 0.05	0.26 ± 0.01
20:3(n-6)	0.64 ± 0.05	0.70 ± 0.06	0.59 ± 0.03	0.41 ± 0.06
20:3(n-3)	0.58 ± 0.05	ND	ND	0.65 ± 0.11
20:4(n-6)	17.94 ± 0.42^{d}	25.04 ± 0.44^{a}	22.98 ± 0.46^{b}	20.10 ± 0.34^{c}
20:5(n-3)	1.28 ± 0.74	tr	ND	ND
22:3	1.24 ± 0.23^{c}	1.84 ± 0.29^{bc}	2.48 ± 0.42^{ab}	3.19 ± 0.27^{a}
22:4(n-6)	0.55 ± 0.05^{b}	0.73 ± 0.06^{a}	0.75 ± 0.05^{a}	0.85 ± 0.02^{a}
22:5(n-3)	0.31 ± 0.01	0.24 ± 0.01	tr	tr
22:6(n-3)	4.93 ± 0.07	5.80 ± 0.42	5.69 ± 0.53	4.05 ± 0.22

[1],[2]Refer to Table 20.
Values expressed as means ± SEM. Differences in horizontal means without a common supercsript are statistically significant ($P \leq 0.05$).

accompanied by higher levels of 20:4(n-6) and 22:4(n-6) in all T_3 groups. The fatty acid profiles for PC and PE (Tables 22 and 23) reflected the fatty acid composition of total PL. Similar elevations in arachidonic acid were observed. Also, in the PE fraction, 16:0 was significantly reduced in all treatment groups and 18:0 was increased in the 0.25 and 0.5 ppm T_3 groups.

Study II: Body weights are shown in Table 24. Animals given 0.05 ppm T_3 in the diet had greater body weights initially and throughout the experiment but this was significant only for week 2.

The weights of all the organs measured were lower for control animals compared to those given T_3 in the diet (Table 25). These differences were statistically significant for the liver, kidney and heart. There was no difference in the length of the colon or the colon index for any of the groups.

The total levels of PC and PE in the colonic mucosa, as well as their relative amounts are presented in Table 26. The results are similar to those reported in the rat liver. PC was significantly increased in all T_3 treated animals. The levels of PE were also higher in treatment groups but this was only significant for 0.25 ppm T_3. The PC to PE ratio was elevated by T_3 feeding with 0.1 ppm T_3 having the greatest

TABLE 22. Effect of T_3 on % fatty acyl composition of PC in rat liver.

Fatty acid	0.0 ppm T_3	0.1 ppm T_3	0.25 ppm T_3	0.5 ppm T_3
14:0	0.42 ± 0.03^{a}	0.18 ± 0.03^{c}	0.20 ± 0.02^{c}	0.30 ± 0.01^{b}
16:0	18.18 ± 0.72^{ab}	13.83 ± 1.16^{c}	15.18 ± 1.08^{bc}	19.01 ± 0.49^{a}
16:1(n-7)	0.60 ± 0.09	0.40 ± 0.01	0.45 ± 0.06	0.61 ± 0.05
18:0	44.13 ± 1.24	41.86 ± 0.64	42.18 ± 1.38	39.86 ± 0.19
18:1(n-9)	3.87 ± 0.15^{b}	4.08 ± 0.07^{b}	4.23 ± 0.33^{b}	5.24 ± 0.22^{a}
18:2(n-6)	6.93 ± 0.47	6.70 ± 0.67	6.98 ± 0.45	6.82 ± 0.14
18:3(n-3)	0.40 ± 0.08	tr[1]	tr	0.28 ± 0.04
20:0	0.50 ± 0.03	tr	tr	0.28 ± 0.04
20:2	0.30 ± 0.03	0.24 ± 0.01	tr	tr
20:3(n-6)	0.53 ± 0.07^{a}	0.62 ± 0.05^{a}	0.54 ± 0.04^{a}	0.38 ± 0.05^{b}
20:3(n-3)	0.82 ± 0.14	ND[2]	ND	0.85 ± 0.03
20:4(n-6)	18.01 ± 0.38^{c}	26.56 ± 2.01^{a}	23.78 ± 0.31^{a}	19.29 ± 0.45^{b}
20:5(n-3)	0.58 ± 0.45	tr	tr	tr
22:3	0.69 ± 0.22^{c}	1.19 ± 0.18^{bc}	1.51 ± 0.19^{b}	2.22 ± 0.17^{a}
22:4(n-6)	0.25 ± 0.02^{c}	0.33 ± 0.04^{b}	0.35 ± 0.02^{b}	0.47 ± 0.02^{a}
22:6(n-3)	3.31 ± 0.09^{a}	3.78 ± 0.26^{a}	3.68 ± 0.22^{a}	2.67 ± 0.12^{b}

[1],[2] Refer to Table 20.
Values expressed as means ± SEM. Differences in horizontal means without a common supercsript are statistically significant ($P \leq 0.05$).

TABLE 23. Effect of T_3 on % fatty acyl composition of PE in rat liver.

Fatty acid	0.0 ppm T_3	0.1 ppm T_3	0.25 ppm T_3	0.5 ppm T_3
14:0	0.53 ± 0.04^a	0.09 ± 0.05^b	0.21 ± 0.03^b	0.10 ± 0.06^b
14:1	0.30 ± 0.09	tr[1]	tr	ND[2]
16:0	21.14 ± 0.57^a	15.83 ± 0.83^b	13.87 ± 0.32^b	14.10 ± 0.89^b
16:1(n-7)	1.00 ± 0.25	0.67 ± 0.11	0.74 ± 0.05	0.56 ± 0.03
18:0	35.37 ± 0.61^b	31.56 ± 0.79^c	37.54 ± 0.57^a	38.05 ± 0.85^a
18:1(n-9)	4.06 ± 0.60^{ab}	4.39 ± 0.23^a	3.02 ± 0.19^{bc}	2.87 ± 0.21^c
18:2(n-6)	3.78 ± 0.27^b	6.62 ± 1.00^a	3.13 ± 0.13^b	2.83 ± 0.13^b
18:3(n-3)	0.66 ± 0.08	ND	ND	ND
20:1	0.29 ± 0.04	tr	tr	ND
20:2	0.60 ± 0.43	0.32 ± 0.03	tr	ND
20:3(n-6)	0.55 ± 0.22	0.68 ± 0.07	0.34 ± 0.03	tr
20:3(n-3)	1.76 ± 0.22	ND	ND	tr
20:4(n-6)	17.55 ± 0.49^b	24.19 ± 1.30^a	25.22 ± 0.67^a	26.57 ± 0.82^a
22:3	1.64 ± 0.33^c	3.16 ± 0.58^b	3.46 ± 0.49^{ab}	4.88 ± 0.46^b
22:4(n-6)	1.20 ± 0.07^b	1.86 ± 0.21^a	1.89 ± 0.09^a	2.21 ± 0.08^a
22:5(n-3)	0.56 ± 0.04	0.38 ± 0.10	0.44 ± 0.02	tr
22:6(n-3)	7.83 ± 0.42^{bc}	9.95 ± 0.69^a	9.51 ± 0.70^{ab}	7.39 ± 0.53^c

[1,2] Refer to Table 20.
Values expressed as means ± SEM. Differences in horizontal means without a common supercsript are statistically significant ($P \leq 0.05$).

TABLE 24. Body weights of rats given various dosages of T_3 in the diet for four weeks.

	Body Weights (g)				
Group	Initial	Week 1	Week 2	Week 3	Week 4
Control	110 ± 5	135 ± 4	161 ± 5	185 ± 5	204 ± 5
0.05 ppm T_3	116 ± 3	145 ± 3[a]	173 ± 3	197 ± 4	217 ± 4
0.1 ppm T_3	105 ± 4	129 ± 4	153 ± 4	182 ± 5	196 ± 5
0.25 ppm T_3	107 ± 2	128 ± 2	154 ± 6	187 ± 7	190 ± 9

Values expressed as means ± SEM. [a]Significantly different from control ($P \leq 0.05$).

TABLE 25. Effect of T_3 on the weight of various organs and the length of the colon in rats.

Group	Weights (g)				Colon length (cm)	Colon index[1]
	Liver	Kidney	Heart	Colon		
Control	8.5 ± 0.4^{b}	0.86 ± 0.04^{c}	0.77 ± 0.03^{c}	1.11 ± 0.04	17.7 ± 0.5	6.4 ± 0.2
0.05 ppm T_3	9.9 ± 0.4^{a}	1.04 ± 0.04^{b}	0.83 ± 0.05^{bc}	1.25 ± 0.07	18.0 ± 0.8	6.9 ± 0.1
0.1 ppm T_3	8.9 ± 0.2^{ab}	1.00 ± 0.03^{b}	0.90 ± 0.06^{b}	1.17 ± 0.04	16.7 ± 0.5	7.0 ± 0.2
0.25 ppm T_3	10.1 ± 0.4^{a}	1.20 ± 0.06^{a}	1.04 ± 0.04^{a}	1.18 ± 0.05	17.9 ± 0.5	6.6 ± 0.2

[1](Weight of colon/length of colon) * 100.
Values expressed as means ± SEM. Means not sharing a common superscript are significantly different from each other ($P \leq 0.05$).

Table 26. Effect of T_3 on the total levels of PC and PE, and their relative amounts in the colonic mucosa of rats.

Group	Total PC (μg/g wet wt liver)	Total PE (μg/g wet wt liver)	PC:PE
Control	79.18 ± 12.55	61.12 ± 7.87	1.30 ± 0.16
0.05 ppm T_3	101.03 ± 9.99[a]	63.84 ± 6.44	1.58 ± 0.20
0.1 ppm T_3	100.94 ± 13.47[a]	58.74 ± 8.43	1.72 ± 0.09[a]
0.25 ppm T_3	121.78 ± 9.89[a]	77.46 ± 4.84[a]	1.57 ± 0.05

Values expressed as means ± SEM. Means sharing a common superscript are not significantly different from each other ($P \leq 0.05$).

effect, as also reported in the liver.

Minor alterations in the fatty acid composition of TG, PL, PC and PE were noted in the colonic mucosa. In the TG fraction (Table 27), the level of 16:0 was higher in all treatment groups while 18:2(n-6) was lower in animals consuming 0.1 and 0.25 ppm T_3. Changes in the fatty acid profile for total PL (Table 28) included elevated levels of 18:0 in treatment groups compared to controls. The most consistent effect of T_3 in the PC fraction (Table 29) was a higher level of 18:0. For PE, 16:1 was lower while 20:4(n-6) was higher for rats fed T_3 (Table 30).

4. Discussion

The main purpose of these studies was to assess the effect of chronic dietary administration of T_3 on plasma cholesterol concentrations and lipid composition of liver and colonic mucosa in rats.

The body weights of the animals given T_3 in the diet were slightly lower than control animals in study I. This could partially be due to the higher dosages of T_3 used in this study or initial variations in body weights. The body weights of the animals were unaffected by the addition of T_3 to the diet in study II. Although the amount of food consumed was not significantly different for any group at any time point,

TABLE 27. Effect of T_3 on % fatty acyl composition of TG in rat mucosa.

Fatty acid	Control	0.05 ppm T_3	0.1 ppm T_3	0.25 ppm T_3
14:0	1.85 ± 0.03^{c}	2.00 ± 0.11^{bc}	2.16 ± 0.08^{ab}	2.37 ± 0.08^{a}
14:1	0.21 ± 0.03	tr[1]	tr	tr
16:0	30.77 ± 0.30^{c}	32.39 ± 0.60^{c}	34.44 ± 0.44^{b}	37.23 ± 0.84^{a}
16:1(n-7)	8.54 ± 0.22^{b}	10.40 ± 0.64^{a}	9.31 ± 0.58^{ab}	9.21 ± 0.24^{ab}
18:0	3.43 ± 0.17^{bc}	2.77 ± 0.43^{c}	3.74 ± 0.15^{b}	4.75 ± 0.21^{a}
18:1(n-9)	32.85 ± 0.60	27.55 ± 6.10	33.97 ± 0.35	34.01 ± 0.62
18:2(n-6)	17.46 ± 0.48^{a}	18.64 ± 3.29^{a}	14.48 ± 0.57^{ab}	9.92 ± 0.44^{b}
18:3(n-3)	0.33 ± 0.01	0.30 ± 0.03	0.21 ± 0.02	tr
20:0	tr	tr	tr	0.24 ± 0.09
20:1	0.37 ± 0.04	0.28 ± 0.08	tr	0.47 ± 0.07
20:2	0.25 ± 0.03	0.24 ± 0.08	tr	tr
20:3(n-6)	tr	tr	tr	tr
20:4(n-6)	0.76 ± 0.15^{a}	0.47 ± 0.04^{b}	0.35 ± 0.03^{b}	0.33 ± 0.03^{b}
22:2	2.52 ± 0.71	1.22 ± 0.39	tr	0.23 ± 0.06
22:3	ND[2]	tr	tr	ND
22:4(n-6)	tr	tr	tr	tr
22:5(n-3)	tr	ND	ND	ND

[1],[2]Refer to Table 20.
Values expressed as means ± SEM. Differences in horizontal means without a common supercsript are statistically significant ($P \leq 0.05$).

TABLE 28. Effect of T_3 on % fatty acyl composition of PL in rat mucosa.

Fatty acid	Control	0.05 ppm T_3	0.1 ppm T_3	0.25 ppm T_3
14:0	1.15 ± 0.06[a]	0.95 ± 0.08[b]	0.88 ± 0.05[b]	1.08 ± 0.06[ab]
16:0	20.29 ± 0.49[b]	18.76 ± 0.70[b]	20.43 ± 0.36[b]	22.94 ± 0.80[a]
16:1(n-7)	2.56 ± 0.25	1.65 ± 0.49	1.75 ± 0.15	2.08 ± 0.16
18:0	21.55 ± 0.38[b]	25.85 ± 1.50[a]	25.35 ± 0.80[a]	26.31 ± 1.33[a]
18:1(n-9)	16.27 ± 0.05	14.71 ± 1.33	14.42 ± 0.48	14.10 ± 0.35
18:2(n-6)	6.07 ± 0.41	6.56 ± 0.20	6.00 ± 0.34	5.96 ± 0.26
18:3(n-3)	0.45 ± 0.12	0.35 ± 0.04	tr[1]	0.30 ± 0.02
20:0	0.56 ± 0.11	0.83 ± 0.07	0.53 ± 0.06	0.62 ± 0.16
20:1	1.94 ± 0.22	0.81 ± 0.27	0.26 ± 0.11	ND[2]
20:2	0.57 ± 0.08	0.58 ± 0.04	0.47 ± 0.05	0.49 ± 0.04
20:3(n-6)	2.09 ± 0.06[a]	1.78 ± 0.10[b]	1.36 ± 0.07[c]	1.67 ± 0.06[b]
20:4(n-6)	16.90 ± 0.57	18.44 ± 0.98	16.01 ± 0.53	18.61 ± 0.43
22:2	ND	tr	tr	ND
22:3	1.73 ± 0.07[b]	1.83 ± 0.14[b]	2.29 ± 0.17[a]	2.40 ± 0.20[a]
22:4(n-6)	5.18 ± 0.06[a]	4.64 ± 0.37[ab]	4.12 ± 0.22[b]	5.28 ± 0.23[a]
22:5(n-3)	tr	tr	tr	tr
22:6(n-3)	2.40 ± 0.09[a]	2.03 ± 0.18[ab]	1.85 ± 0.14[b]	1.88 ± 0.10[b]

[1],[2]Refer to Table 20.
Values expressed as means ± SEM. Differences in horizontal means without a common supercsript are statistically significant ($P \leq 0.05$).

TABLE 29. Effect of T_3 on % fatty acyl composition of PC in rat mucosa.

Fatty acid	Control	0.05 ppm T_3	0.1 ppm T_3	0.25 ppm T_3
14:0	2.20 ± 0.16^{a}	1.56 ± 0.05^{b}	1.49 ± 0.21^{b}	1.12 ± 0.14^{b}
16:0	32.51 ± 0.69	30.88 ± 1.53	30.89 ± 0.69	30.33 ± 0.43
16:1(n-7)	3.47 ± 0.17^{a}	2.84 ± 0.34^{a}	3.52 ± 0.78^{a}	1.46 ± 0.13^{b}
18:0	25.47 ± 0.58^{b}	28.06 ± 1.00^{ab}	31.23 ± 1.28^{a}	29.92 ± 1.12^{a}
18:1(n-9)	15.19 ± 0.55	15.41 ± 0.92	13.25 ± 1.08	14.29 ± 0.41
18:2(n-6)	4.90 ± 0.22	5.58 ± 0.36	4.40 ± 0.37	5.32 ± 0.44
18:3(n-3)	tr[1]	0.31 ± 0.10	tr	0.25 ± 0.02
20:0	1.05 ± 0.19	0.82 ± 0.04	1.18 ± 0.66	0.53 ± 0.13
20:1	1.62 ± 0.22^{a}	1.54 ± 0.28^{ab}	0.67 ± 0.39^{bc}	0.42 ± 0.24^{c}
20:2	0.23 ± 0.15^{b}	0.59 ± 0.07^{a}	0.55 ± 0.06^{a}	0.57 ± 0.06^{a}
20:3(n-6)	0.95 ± 0.08^{b}	1.42 ± 0.09^{a}	1.19 ± 0.05^{a}	1.36 ± 0.07^{a}
20:4(n-6)	9.48 ± 0.94	8.09 ± 0.43	8.19 ± 0.51	10.56± 0.35
22:2	tr	tr	ND[2]	ND
22:3	0.36 ± 0.15^{b}	0.59 ± 0.04^{ab}	0.79 ± 0.27^{ab}	1.01 ± 0.11^{a}
22:4(n-6)	2.00 ± 0.09^{ab}	1.63 ± 0.06^{c}	1.78 ± 0.12^{bc}	2.28 ± 0.13^{a}
22:6(n-3)	0.37 ± 0.16	0.63 ± 0.05	0.68 ± 0.21	0.59 ± 0.05

[1],[2]Refer to Table 20.
Values expressed as means ± SEM. Differences in horizontal means without a common supercsript are statistically significant ($P \leq 0.05$).

TABLE 30. Effect of T_3 on % fatty acyl composition of PE in rat mucosa.

Fatty acid	Control	0.05 ppm T_3	0.1 ppm T_3	0.25 ppm T_3
14:0	0.99 ± 0.13[a]	1.11± 0.28[a]	0.64 ± 0.04[ab]	0.44 ± 0.07[b]
16:0	8.31 ± 1.05	7.65 ± 0.55	8.38 ± 1.04	6.26 ± 0.20
16:1(n-7)	5.90 ± 0.53[a]	3.62 ± 0.11[b]	3.67 ± 0.66[b]	2.77 ± 0.26[b]
18:0	27.25 ± 0.62	28.94 ± 0.92	28.54 ± 0.82	27.89 ± 0.73
18:1(n-9)	12.83 ± 0.96	10.51 ± 0.87	12.47 ± 1.12	11.72 ± 0.97
18:2(n-6)	3.42 ± 0.12	2.85 ± 0.31	3.28 ± 0.44	2.52 ± 0.18
18:3(n-3)	0.33 ± 0.15	0.38 ± 0.11	tr[1]	0.48 ± 0.08
20:0	0.43 ± 0.22	0.70 ± 0.29	0.61 ± 0.33	0.80 ± 0.07
20:1	1.12 ± 0.08	ND[2]	ND	tr
20:2	tr	tr	tr	tr
20:3(n-6)	1.22 ± 0.04	1.51 ± 0.10	1.25 ± 0.13	1.36 ± 0.09
20:4(n-6)	25.36 ± 0.90[c]	28.50 ± 0.23[ab]	26.87 ± 1.38[bc]	30.49 ± 0.36[a]
22:2	tr	ND	ND	ND
22:3	0.38 ± 0.38[b]	2.86 ± 0.28[a]	3.16 ± 0.32[a]	3.67 ± 0.24[a]
22:4(n-6)	8.52 ± 0.24	7.45 ± 0.18	7.57 ± 0.23	8.30 ± 0.89
22:5(n-3)	0.33 ± 0.09	0.27 ± 0.08	0.21 ± 0.10	tr
22:6(n-3)	3.27 ± 0.16	3.54 ± 0.44	3.18 ± 0.27	3.13 ± 0.16

[1],[2]Refer to Table 20.

Values expressed as means ± SEM. Differences in horizontal means without a common supercsript are statistically significant ($P \leq 0.05$).

a trend was evident towards increased food consumption for animals eating diets containing T_3.

Feeding T_3 in the diet is a mode of treatment which has been successfully used by other researchers to elevate circulating T_3 levels (Perry et al., 1988; Woods and Woodward, 1991). Dietary T_3 resulted in a dose dependent increase in serum T_3 concentrations and this was accompanied by a reduction in serum cholesterol level. This observation is consistent with the findings of other investigators who reported lower plasma cholesterol content in hyperthyroid rats as compared to euthyroid (Ruggiero et al., 1990). It has also been demonstrated that hypothyroidism is associated with enhanced plasma cholesterol levels (Ruggiero, 1987).

Interestingly, several studies have shown an inverse relation between serum cholesterol and cancer incidence (Isles et al., 1989; Schatskin et al., 1987; Trichopoulou et al., 1992; Williams et al., 1981; Winawer et al., 1990). This relationship has been most consistently demonstrated for large bowel cancer. While several workers have suggested that low serum cholesterol levels are a consequence of the disease and represent a preclinical cancer effect (Neugut et al., 1986; Nomura et al., 1991), other studies do not support this conclusion. Winawer et al. (1990) reported a gradual decrease in serum cholesterol during the ten years prior to the

clinical appearance of cancer, indicating that declining cholesterol levels preceded the development of cancer.

T_3 increased the growth of various tissues in the rat. The weights of the liver, kidney and heart were greater in animals given diets containing T_3 compared to controls. This anabolic effect could be due to a general increase in protein and RNA synthesis (Johnson, 1987). The colon weight, length and index were unaltered by T_3.

The results of these studies demonstrate that T_3 affects the distribution and pattern of lipids in the liver and colonic mucosa of the rat. PC, and to a lesser extent PE, tended to be elevated by T_3 at all dose levels in both the liver and colonic tissues. The ratio of PC to PE was higher in animals given 0.1 ppm T_3 in the diet. Most if not all PL molecules are present in biomembranes (Fourcans, 1974). Therefore, changes in the PL headgroups, associated with altered packing arrangement of the PL molecules may have a significant effect on the permeability and function of the membrane *in vivo* (Sandermann, 1978; Stubbs and Smith, 1984). Generally, elevated levels of PC accompanied by a higher PC:PE ratio increases membrane fluidity due to the more spacious distribution of the larger PC head group (McElhaney, 1984).

The fluidity and permeability of biological membranes also

depends on the nature of the acyl chains (Murphy, 1990; Stubbs and Smith, 1984). In the present studies, the fatty acid profile of total PL as well as PC and PE in the liver was markedly altered by T_3. There were lower levels of the shorter fatty acyl chain lengths, such as 16:0, accompanied by higher levels of 20:4(n-6) and 22:4(n-6). These results indicate that T_3 stimulated chain elongation and desaturation. The fatty acid profile of PE in the colonic mucosa demonstrated similar changes. Other researchers have supported hyperthyroid induced increases in fatty acid unsaturation and chain elongation synthesis in the mitochondria of rat liver and kidney (Morini et al., 1991; Ruggiero et al., 1984). These changes are generally associated with a more fluid and permeable membrane (Stubbs and Smith, 1984).

The possible increase in membrane fluidity resulting from elevated levels of PC and fatty acyl unsaturation could have important implications to the carcinogenic process. It has been hypothesized that cancer results from changes in the uptake mechanisms of cells caused by changes in the cell membrane fluidity (Holley, 1972). Furthermore, other researchers have demonstrated a similar increase in the PC:PE ratio in the colonic mucosa of mice fed a known tumour promoting diet (Robblee et al., 1988).

Altered membrane structure can also influence the activity of lipid dependent enzymes (Sandermann, 1978). Changes in membrane lipid composition and thus membrane fluidity have been shown to influence the conformation of the active site of membrane-associated enzymes (McMurchie and Raison, 1979). Plasma membranes contain a number of thyroid sensitive enzymes, such as adenylate cyclase, $Na^{+}K^{+}$-ATPase and $CA2^{+}$-ATPase, that are responsive to manipulations in membrane lipid composition (Hoch, 1988). Perhaps changes in membrane fluidity is the manner in which the activities of many membrane-associated enzymes are modified by thyroid hormones (Ruggiero et al., 1984). Lenaz et al. (1983) have suggested that various pathological states, including malignancies, may originate from impaired activity of enzymes, carriers or receptors caused by disorganized lipid-protein interactions and altered membrane fluidity.

In the present studies, T_3 elevated the levels of arachidonic acid in the liver and colonic tissues. The lower levels of 18:2(n-6) in the TG fraction may be due to mobilization of linoleic acid followed by conversion into arachidonic acid and incorporation into PL. The rise in arachidonic acid in the T_3 treated groups has important implications to cancer development. Studies have reported a considerable increase in the content of arachidonic acid in colorectal tumours compared to noncancerous tissue in both humans (Neoptolemos et al.,

1991) and rats (Nicholson et al., 1991). Furthermore, the rise in arachidonic acid could indicate enhanced eicosanoid synthesis of which 20:4(n-6) is the predominant precursor (Baker, 1990). The eicosanoids, including prostaglandins (PG), thromboxanes (TX) and leukotrienes (LT), are recognized to play a role in signal transduction and are important regulators of cell function (Merrill, 1989; Shimizu and Wolfe, 1990). The possible role for PG and other eicosanoids in controlling cell proliferation and neoplasia has been recognized for some time (Jaffe, 1974). In fact, the levels of PGE_2 and TXB_2 have been shown to be higher in cancerous tissue of the colon as compared to noncancerous mucosa (Yamaguchi et al., 1991).

The findings of the present study that T_3 is capable of modifying lipid composition of liver and colonic tissue, especially by altering the polar head groups and increasing unsaturation of membranes, may have important biological implications.

Chapter VII

GENERAL DISCUSSION

A. T_3 and Colon Carcinogenesis

This research employed aberrant crypt foci (ACF) as the biological endpoint to test the hypothesis that 3,5,3'-triiodothyronine (T_3), the biologically active thyroid hormone, plays a role in the development of cancer of the colon. This hypothesis was based on the contention that dietary intake and composition, well known modulators of carcinogenesis, alter the metabolism of thyroid hormones. Caloric excess, reported to enhance cancer risk and incidence, is associated with elevated levels of serum T_3. Given that thyroid hormones regulate numerous metabolic functions in most mammalian tissues and influence the processes involved in growth and differentiation, it was of interest to investigate the effect of elevated levels of T_3 on the induction and growth of ACF, putative preneoplastic lesions, in the colons of CF1 mice and Sprague-Dawley rats, as well as any proliferative changes in the colonic epithelium, a proposed risk marker of colon carcinogenesis. Furthermore, it was important to assess the affect of T_3 on tissue lipid composition due to the reported ability of thyroid hormones to alter the activities of various membrane associated enzymes involved in metabolism and cell regulation.

Although the physiological actions of thyroid hormones have been extensively studied, a review of the literature revealed a general lack of information on the use of whole organisms in the study of the long term effects of thyroid hormones on a chronic disease such as cancer. Furthermore, as outlined in the previous chapters, the reported modulating effect of nutrition on thyroid status and the known physiological effects of thyroid hormones, led to the hypothesis and subsequent investigation of the possible cancer modulating effect of T_3. However, there were uncertainties with respect to dosage levels and choice of animal model. Therefore, the studies reported herein were designed to provide a framework on which to base further studies investigating the role of thyroid hormone on cancer development.

Short term continuous feeding of a diet containing various dosages of T_3 increased the induction and crypt multiplicity of ACF in the murine colon. This effect was most prominent when T_3 was administered at a dose level of 0.5 to 2.0 ppm simultaneously with AOM, as opposed to one week prior to or one week following AOM exposure, suggesting that T_3 and AOM cooperated in these events. This observation is supported by the findings of other workers who reported that the rate of carcinogen induced transformation *in vitro* is most enhanced when the cells are exposed to T_3 at the time of initiation (Borek et al., 1985; Borek et al., 1983). T_3 also induced a

hyperplastic response in the mouse crypts regardless of the feeding schedule, suggesting that increased cell proliferation may not be the main causative factor associated with enhanced development of ACF.

Further studies were conducted to evaluate the possibility that Sprague-Dawley rats, the animal model extensively used in experimental colon carcinogenesis, can be utilized to investigate the cancer modulating effect of T_3. Contrary, to the finding in the murine colon, T_3 did not increase the number and growth of ACF whether it was added to the diet during initiation or one week later. T_3 did however stimulate cell production and accumulation (hyperplasia) in the rat colonic crypts. Furthermore, it was demonstrated that CF1 mice differ a great deal from Sprague-Dawley rats with respect to tolerance to T_3 treatment. For example, carcinogen treated mice were able to tolerate up to 2.0 ppm T_3 in the diet whereas rats had difficulty tolerating a dose level of 0.5 ppm T_3. A preliminary study with rats using 1.0 ppm T_3 in the diet resulted in death of the animals within three weeks of commencing the feeding. These findings suggest that rats and mice may differ in their gut uptake and metabolism of T_3.

The inconsistent effect of T_3 on early neoplastic events in the rat and mouse colon raises some interesting questions regarding the species and organ specific action of T_3, as well

as the differences in thyroid hormone metabolism in various models. These results demonstrate that biological systems may vary in their responsiveness and sensitivity to T_3. Furthermore, the observation that T_4 enhances the development of colonic tumours in rats (Iishi et al., 1992) and accelerates the progression of implanted cancer cells in mice (Kumar et al., 1979), suggests that T_4 and T_3 may need to be studied independently as modulators of carcinogenic events. There is also the possibility that T_3 may have an effect on the later stages of colon cancer development

In addition to exerting a regulatory effect on cell growth in the colonic epithelium, T_3 induced significant changes in membrane PL composition in liver and colonic tissue. PC levels were markedly elevated while PE was slightly higher in T_3 treated animals. These changes tended to be accompanied by an increase in the PC to PE ratio. The fatty acid profiles of the PLs indicated a trend towards elongation and unsaturation of the fatty acids chains. Alterations in membrane structure and thus function are postulated to play a role in the development of various malignancies (Lenaz et al., 1983). T_3 feeding also caused a reduction in serum cholesterol levels, which in turn have been associated with an increased incidence of colon cancer (Schatzkin et al., 1987; Winawer et al., 1990).

The findings of the studies presented in this thesis suggest that the potential role of T_3 in modulating cancer processes may differ in various species. The results indicate that T_3 increases the number and growth of ACF, putative preneoplastic lesions of colon cancer, in CF1 mice but not in Sprague-Dawley rats. Furthermore, the results indicate that enhanced cellular proliferation is not necessarily the main mechanism by which T_3 exerts its effect on the development of ACF. Given that T_3 stimulated significant alterations in the lipid metabolism and composition of rat liver and colonic tissues, it is possible that the cancer modulating effect of T_3 is mediated by its action on various membranes and associated systems.

B. Major Findings and Implications to Humans

The findings of this dissertation have provided fundamental information on the feasibility of using animal models to study the effect of T_3 on the early stages of colon cancer development. This research has established the dose levels of T_3 tolerated by the murine and rat model. CF1 mice were able to tolerate up to 2.0 ppm T_3 in their diet whereas Sprague-Dawley rats appeared to be more sensitive and tolerated T_3 feeding up to a dosage of 0.25 ppm in their diet. T_3 at a dose level of 0.5 ppm resulted in toxic symptoms and 1.0 ppm resulted in mortality within three weeks in carcinogen treated rats. This research has also demonstrated that with respect

to the effect of T_3 on the induction and growth of ACF, mice respond differently than rats. Furthermore, this work has provided information on the possible metabolic effects of T_3 at the tissue level including hyperplastic changes in the colonic epithelium and marked alterations in the composition of the membranous polar head groups and acyl chains. In addition, it has established that carcinogen treated rats appear to tolerate the level of T_3 differently than those not treated with AOM.

The finding that T_3 may affect early carcinogenic events in various animal species has important implications to humans. First of all, T_3 levels in humans can be altered by dietary factors, which themselves have been shown to modify cancer incidence in animal models. Excess caloric consumption, postulated to be a risk factor for cancer development (Lyon et al., 1987), is accompanied by enhanced circulating levels of the potent thyroid hormone, T_3, in humans. In view of the metabolic and growth regulatory functions of thyroid hormones, one can speculate that dietary modulation of tumour growth and development may involve alterations in thyroid hormone metabolism.

Further relevance of this research to humans lies in the fact that thyroid disorders are amongst the most common afflictions involving the endocrine system. Hyperthyroidism and

hypothyroidism associated with abnormally high or low circulating levels of the thyroid hormones, respectively, is a common metabolic disorder in people. Whether individuals with subtle elevated levels of T_3 have an increased risk of cancer development or have a greater incidence of cancer has not yet been determined.

C. Future Perspectives

The findings in this dissertation provide a framework for future studies to be carried out in animal models. Furthermore, this research provides impetus to assess the risk of cancer development in individuals with varying thyroid status. However, in view of the main focus of this research, it is imperative to elaborate on how animal models can be utilized for further studies. For instance, investigation into the potential role of T_3 as a modulator of the cancer process requires a better understanding of the multitude of thyroid hormone effects. Although significant progress has been made in identifying the mechanisms by which thyroid hormones exert a variety of physiological actions, there is still a great deal that remains to be understood, particularly with respect to the specificity of thyroid hormone action. Clarification of the genomic and nongenomic effects, and their relative importance to various metabolic and growth effects is necessary. Further research is required to determine whether the nucleus is the primary site of action involving altered

gene expression and protein synthesis or whether actions are carried out at several primary sites within the target cell including the plasma membrane, nucleus, and mitochondria.

In addition, studies must be directed at identifying the various species and organs which are sensitive to the diverse effects of the thyroid hormones. Future investigations regarding the role of thyroid hormones in the genesis of cancer of various sites including the colon, must focus on the development of a suitable and appropriate model for the *in vivo* study of the hormone's effects in whole organisms. The mechanism by which thyroid hormones may alter neoplastic processes also needs to be addressed. Whether the hormones have a direct effect or act indirectly through the mediation of other hormones or nutritional factors remains to be established. Furthermore, various protocols need to be implemented to determine which stage or stages of the cancer process - initiation, promotion or progression - are influenced by thyroid hormones. Most importantly, the interrelationship between nutritional status and thyroid hormone metabolism and biological potency remains a challenging field for future exploration.

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Chapter IX

APPENDICES

A. Composition of AIN-76 Mineral and Vitamin Mixtures

AIN-76 Mineral Mixture

Composition:	grams
calcium phosphate dibasic	500.00
sodium chloride	74.00
potassium citrate monohydrate	220.00
potassium sulphate	52.00
magnesium oxide	24.00
manganese carbonate (43-48% Mn)	3.50
ferric citrate (16-17% Fe)	6.00
zinc carbonate (70% ZnO)	1.60
cupric carbonate (53-55% Cu)	0.30
potassium iodate	0.01
sodium selenite	0.01
chromium potassium sulphate	0.55
sucrose, finely powered	118.00

AIN-76 Vitamin Mixture

Composition:	per kg of mixture
thiamine hydrochloride	600.0 mg
riboflavin	600.0 mg
pyridoxine hydrochloride	700.0 mg
nicotinic acid	3.0 g
D-calcium pantothenate	1.6 mg
folic acid	200.0 mg
D-biotin	20.0 g
cyanocobolamin (vitamin B_{12})	1.0 mg
retinyl palmitate (vitamin A) pre-mix (250,000 IU/g)	1.6 g
DL-α-tocopherol acetate (vitamin E) pre-mix (250IU/g)	20.0 g
cholecalciferol (vitamin D_3) (400,000 IU/g)	250.0 mg
menaquinone (vitamin K)	5.0 mg
sucrose, finely powdered	972.9 g

B: Anti-Bromodeoxyuridine Staining Procedure

Immunoperoxidase Procedure

1. Depariffinize slides:
   xylene: 5 min - repeat 3x in fresh xylene
   100% ethanol: 2 min - repeat 2x
   95% ethanol: 2 min
   70% ethanol: 2 min
   dH_2O: 5 min

2. Hydrolyse slides in 2N HCl for 1.5 hr.

3. Place in 0.1M $Na_2B_4O_7$ for 5 min to stop reaction.

4. Wash slides in phosphate buffered saline (PBS) 3x. Carefully blot excess solution from the slide and around the tissue.

5. Add Signet kit reagent #2 to tissue (normal goat serum blocking agent). Place in humid chamber for 20 min at ambient temperature.

6. Wash slides in fresh PBS 3x. Blot.

7. Add anti-BUdR MOab (1:40 dilution). Place in humid chamber for 1 hr at ambient temperature.

8. Wash slides in fresh PBS 3x. Blot.

9. Add Signet kit reagent #4 to tissue (linking agent - goat anti-mouse Ab). Place in humid chamber for 20 min at ambient temperature.

10. Wash slides in fresh PBS 3x. Blot.

11. Add Signet kit reagent #5 to tissue (labelling agent - mouse IgG peroxidase). Place in humid chamber for 20 at ambient temperature.

12. Wash slides in fresh PBS 3x (no need to blot).

13. 3,3' Diaminobenzidine Tetrahydrochloride (DAB) staining:
   a. add 1.25 ml of stock DAB to 200 ml PBS.
   b. add 125 μl 30% H_2O_2 and immediately pour over slides.
   c. immerse slides for 5 min.

14. Rinse slides in dH_2O 2x.

15. Dip or immerse slides in haematoxylin stain for 3 sec.

16. Rinse slides in dH_2O 2x.

17. Dehydrate slides:
    70% ethanol - 1 min
    95% ethanol - 1 min
    100% ethanol - 1 min
    xylene - 2 min

18. Apply permount and cover glass.

C: Immuchem ^{125}I Triiodothyronine Radioimmunoassay

Calculations

Note: A NON-SPECIFIC BINDING should be utilized in the calculations. The NSB is routinely found to be 1.8% of the total amount of radioactivity of 1.0 ml of T_3-^{125}I (the total tube).

A. Take the average counts of all duplicate tube (samples and standards). Subtract the averaged blank (NSB) counts from the averages obtained above. This yields the corrected values. Divide the corrected values by the corrected zero standard value to obtain the percent bound.

B. Formula

$$\%B/Bo = \frac{\text{CPM (sample)} - \text{CPM (blank NSB)}}{\text{CPM (0 standard)} - \text{CPM (blank NSB)}} \times 100$$

CPM = Average counts of duplicates.

Sample = Particular sample or standard being calculated.

Blank (NSB) = Blank tube (also known as non-specific binding tube).

0 standard = 0 tube (also known as 100% binding tube.

C. Construct a plot of percent bound vs the concentration of the standard starting with 25 ng/dL point. This yields the standard curve.

D. Using the standard curve, determine the T_3 concentration for each sample.