1130

DIET, HORMONES AND NMU-INDUCED RAT MAMMARY CANCER

VOEDING, HORMONEN EN DOOR NMU GEINDUCEERDE MAMMATUMOREN BIJ DE RAT

(met een samenvatting in het Nederlands)

PROEFSCHRIFT

Ter verkrijging van de graad van
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Rijksuniversiteit te Utrecht,
op gezag van de Rector Magnificus,
Prof. Dr. J.A. van Ginkel,
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GIJSBERTUS SILVESTER JOZEF BUNNIK

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Promotoren: Prof.Dr. J.H.H.Thijssen
Prof.Dr. R.J.J.Hermus

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STELLINGEN

1. In alle onderzoekingen waarbij endocriene parameters betrokken zijn, dient rekening gehouden te worden met de invloed van de proefomstandigheden op die parameters.

2. Het veronderstelde verhogend effect van voedingsvet op plasmagehalten van prolactine en estradiol in gezonde en tumordragende dieren, dient sterk in twijfel getrokken te worden.

3. De hypothese dat voedingsvet een verhoogde mammatumorrespons bij de rat bevordert via een verhoging van plasmagehaltes van prolactine en estradiol wordt vooralsnog gesteund door vals positieve argumenten.

4. Endocriene factoren spelen zowel een rol bij de initiatie als bij de promotie, de progressie en de prognose van borstkanker.

5. Voeding heeft een directe invloed op de ontwikkeling van weefsels en mede daardoor kan voeding een belangrijke rol spelen in de carcinogenese.

6. Een lagere vetconsumptie en een hogere consumptie van verse groenten en vers fruit, alsook een hogere vezel-inname zou wel eens een geweldig potentieel voor de preventie van meerdere soorten kanker kunnen bieden.

7. De gecompliceerde interacties tussen voedingsfactoren en orgaansystemen rechtvaardigen nader fundamenteel en toegepast onderzoek om tot opheldering van die mechanismen te kunnen komen.

8. Zo men al zou willen overgaan tot het geven van algemene voedingsaanbevelingen ter preventie van kanker, dan dient men zich tevens af te vragen of deze opwegen tegen mogelijke nadelen van sociaal en economische aard, of dat zij zelfs gezondheidsrisiko's met zich mee brengen in relatie tot leeftijdgerelateerde ziekten anders dan kanker.

9. Voortgaande identificatie van oncogenen, in het bijzonder van die welke geactiveerd zijn in borsttumoren, kan bijdragen aan inzicht in de mechanismen die ten grondslag liggen aan borstkanker en de invloed die hormonen daarop hebben.

10. Het is mogelijk dat de mate van ontwikkeling en differentiatie van mammaepitheel aanleiding geeft tot onbedoelde initiële verschillen in carcinogeniteitsstudies waarin proefdieren vanaf speenleeftijd verschillende rantsoenen nuttigen.

11. Stellingen hebben een bijzondere ondersteunende betekenis voor bouwvakkers en promovendi.

12. Scheidsrechters bij wedstrijdsporten zijn zo'n noodzakelijk kwaad, dat het noodzakelijk is tijdens sportwedstrijden niet kwaad op ze te worden.

13. De overwaardering van de strafcorner in hockeywedstrijden kan geneutraliseerd worden door een opwaardering van velddoelpunten.

14. Bij het streven naar kwaliteitsverhoging van wetenschappelijk onderzoek is de toenemende vermindering van het onderzoeksbudget geen juiste oplossing.

G.S.J.Bunnik, Utrecht, 29 november 1988.
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Ter dankbare herinnering aan mijn moeder,
voor Pa, Ireen, Bastiaan en Reinier.

VOORWOORD

Met het afronden van dit proefschrift zet ik een punt achter mijn formele universitaire opleiding. Met plezier kijk ik terug op een leerzame en tegelijk boeiende tijd. Met het tot stand komen van dit proefschrift wil ik dan ook iedereen bedanken die mij onderweg geholpen heeft.
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CHAPTER 1

GENERAL INTRODUCTION

Mortality due to neoplastic proliferation of endocrine sensitive tissue is relatively high in the Western world. Breast cancer is the leading cause of mortality among women in many countries, despite advances in early detection techniques and treatment. In the Netherlands, for example, about 20% of total cancer mortality among women is attributed to cancer of the breast. Epidemiological (1-4) and experimental (5-8) studies have indicated that endocrine factors as well as life-style factors are involved in the etiology of breast cancer. Among the life-style factors, dietary habits are thought to be important variables in the pathogenesis of several types of cancer. Besides breast cancer (9), dietary habits are also believed to be involved in prostate cancer (10), colon cancer (11), stomach cancer (12), lung cancer (13) and pancreas cancer (14). Nutritional factors normally do not act as initiators but mainly modify the carcinogenic process initiated by other agents. Therefore, society likes to have preventive dietary guidelines that may help to reduce the risk of cancer in a given population. Before firm recommendations can be given the reliability of any relations between dietary constituents and cancer must be established. Careful evaluations have hence been carried out by the American Committee on Diet, Nutrition and Cancer (Commission on Life Sciences; National Research Council) in 1982 (15) and by the Dutch "Voedingsraad" (Dutch Nutrition Council; committtee Nutrition and Cancer) in 1986 (16). Both of them confirm positive associations between:

1. dietary fat intake and cancer of the breast and of the colon,
2. intake of nitrate and nitrite, and cancer of the stomach,
3. alcohol intake and cancer of the larynx and of the esophagus.

Negative associations are assumed for:

1. dietary selenium intake and cancer of the breast, and
2. vitamin A and β-carotene intake and cancer of the lung and of the bladder.

For most other interactions between diet and carcinogenesis as discussed in the literature, either the results were judged insufficiently consistent or experimental data were too limited to allow firm conclusions, despite various reports by leading scientists. The whole complex of causes and consequences is

hard to support with sound arguments. One must consider whether the advantages of dietary recommendations are strong enough to counterbalance possible social or economic disadvantages or even health risks such as negative influences on age-related diseases other than cancer.

Dietary habits are among the well known life-style factors. The geographic distribution of breast cancer reveals that tumor incidence is higher in western European industrialized countries than in less industrialized countries in Asia and Africa. Dietary habits in industrialized countries are best characterized by excessive energy intake (overnutrition), high intake of dietary fat (mainly of animal origin), low intake of carbohydrates, high intake of animal protein, and low intake of dietary fiber. This is opposite to less-industrialized countries where undernourishment, low fat, high carbohydrate, high vegetable protein, and many fiber-containing products are characteristic of the diet. Epidemiological studies show that part of the international variation in breast cancer incidence may be explained by differences in fat consumption patterns. However, consumption of fat is closely linked with other parameters of the diet, such as total energy intake, the ratio between saturated and unsaturated fatty acids, animal protein, and several minor dietary constituents. The content and type of fat in the diet cannot fully explain the geographic variation, but a causal relationship must be looked for.

Interestingly, most of the cancer types associated with dietary influences are hormone-related, i.e. breast, prostate, colon and pancreas cancer. The nature of the initiating agents is uncertain but it may be worthwhile to consider dietary factors in the prevention of cancer. This may be particularly relevant as it is suggested that nutritional factors influence the post-initiation phase of carcinogenesis. An adjusted diet might then support endocrinetherapy, chemotherapy or immunotherapy, or it might speed up convalescence after surgery.

There is evidence from epidemiological studies and from experimental research that steroid hormones, especially estrogens, progestagens and androgens, as well as prolactin are involved in the etiology of human breast cancer (8,17). Both normal and neoplastic breast tissue are to some extent hormone-dependent. However, the numerous studies reporting about plasma levels of these hormones are controversial and inconclusive. Related studies in animal systems are not unequivocally clear either.

Both the age at menarche and the age at the onset of menopause are hormone-related risk factors for breast cancer. These factors are also dependent on the amount of body fat and hence most likely on total energy intake. As a consequence, it is postulated that diet, predominantly fat intake, may influence or even cause the development of hormone-dependent tumors by changing hormone metabolism.

Hormones exert their biological effects -among others- through stimulation of growth of their target tissue via interaction with specific binding proteins, called 'receptors'. Plasma hormone levels reflect the systemic result of synthesis and release of hormones from the endocrine tissues and elimination from the blood compartment. It is worthwhile to investigate whether and how dietary factors influence endocrine parameters relevant to breast carcinogenesis.

This thesis deals with an experimental approach to test the postulated relation "diet, hormones and breast cancer". The animal model was chosen with the background perception that each experimental method has its limitations and the results of a single method need to be confirmed by another one relevant to man. Moreover, it is necessary to learn and understand working mechanisms since this knowledge may help optimize effects of dietary components to cure and/or prevent cancer and to avoid any unwanted side-effects of dietary modification. The purpose of the studies was to investigate systematically the influence of selected dietary factors on the endocrine system in sofar it is related to mammary carcinogenesis. The rat was selected as the experimental animal since a considerable amount of knowledge on nutritional requirements and endocrinology as well as on mammary carcinogenesis in this species is available. Experiments have been performed in both untreated animals and in Fischer F344 rats treated with a chemical carcinogen (N-nitroso-N-methylurea) to study tumor development. The studies were part of the research program on diet and cancer of the TNO-CIVO Toxicology and Nutrition Institute (18,19).

The aim of the present studies was essentially twofold:

(1) to test the hypothesis that dietary fat and protein influence development of NMU-induced mammary carcinogenesis in rats, and

(2) to assess whether these influences are mediated by altered hormone levels of prolactin, estradiol-17β, progesterone, and corticosterone, and/or by altered steroid hormone receptor content in mammary tumor cytosol.

An extensive survey of the literature on the stage of research on nutrition, hormones and breast cancer is presented in Chapter 2.

Chapter 3 deals with the question whether both type and content of fat in the diet are capable of influencing plasma levels of prolactin, estradiol-17β, progesterone and corticosterone in healthy non-tumor-bearing animals. The influence of the method of blood sampling on plasma hormone levels was investigated as well.

In Chapter 4 the interaction between contents of linoleic acid and fat in the diet on NMU-induced mammary cancer in F344 rats is described.

Chapter 5 deals with the interactive effects of dietary fat and linoleic acid on plasma prolactin, 17β-estradiol, progesterone and corticosterone, and on steroid hormone receptors in NMU-induced mammary cancer in F344 rats.

Chapter 6 reports on the interactive effects of type and content of dietary protein on plasma levels of prolactin and estradiol-17β and on NMU-induced mammary carcinogenesis in F344 rats.

Finally, the general discussion is followed by a final conclusion, a perspective, and remarks on recommendations to the public, presented in Chapter 7.

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CHAPTER 2

NUTRITION, HORMONES AND BREAST CANCER: A SURVEY OF THE LITERATURE

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CONTENTS :

List of abbreviations used

AR	androgen receptor
BC	breast cancer
BHT	butylated hydroxy toluene
c-AMP	cyclic adenosine monophosphate
DHT	dihydrotestosterone
DMBA	7,12-dimethylbenzo(a)anthracene
E_1	estrone
E_1S	estrone-sulphate
E_2	estradiol
E_3	estriol
EGF	epidermal growth factor
ER	estrogen receptor
FGF	fibroblast growth factor
GF	growth factor
GH	growth hormone
HF	high fat
IGF	insulin-like growth factor
LA	linoleic acid
LF	low fat
MCA	3-methylcholanthrene
NGF	nerve growth factor
NMU	N-methylnitrosurea
2-OH-E_1	2-hydroxy-estrone
PDGF	plateled derived growth factor
PR	progesterone receptor
Prog	progesterone
Prol	prolactin
PUFA	poly unsaturated fatty acid
SAFA	saturated fatty acid
SD rat	Sprague Dawley rat
Se	selenium
SHBG	sex hormone binding globulin
T	testosterone
TGFα	transforming growth factor α
TGFβ	transforming growth factor β

1. INTRODUCTION

Cancer and cardiovascular disease are causes of human mortality in industrialized countries. Mortality due to neoplastic proliferation of breast tissue is high in the Western world (1). Despite advances in treatment and early detection techniques, breast cancer (BC) still remains the leading cause of cancer death among women in many countries. In the Netherlands, about 20% of total cancer mortality in women is attributed to cancer of the breast. Both normal and neoplastic breast tissue are to some extent hormone-dependent. Therefore the endocrine system is assumed to be involved in the etiology of this type of cancer. As will be demonstrated later, epidemiological data indicate that life-style factors, in particular dietary habits, are associated with BC. A successful approach to prevent cancer may be the identification and subsequent avoidance of dietary carcinogenic and cancer-enhancing factors. Several leading scientists have estimated that such an approach offers a potential for reducing cancer incidence by as much as 40-70%, based on the large differences in cancer incidence in the world (2-4).

2. EPIDEMIOLOGY

Particularly in industrialized countries mortality from BC has either remained steady or continued to increase (5). The incidence of BC varies from country to country. Collection of data on incidence and mortality from cancer at different sites of the body (6), and on the food available in different countries, made it possible to study correlations between the characteristics of national food supplies and the occurrence of different types of cancer. Some clear outlines of BC epidemiology have been presented recently (7,8).

Briefly, mean findings on the epidemiology of BC include:

Geographic variation. For BC the incidence and mortality rates are five to six times higher in North America and Northern Europe than in most Asian and indigenous African populations. Populations of Southern and Central America and Eastern Europe have rates intermediate between those of Asia and North America (9).

Studies of migrant populations. Studies of migrants from low-risk areas (Asia) to the USA and their descendants have shown that for cancers of the

colon, breast, pancreas and prostate the migrating population tends to exhibit the risk characteristic of the host country. Environmental factors, in particular life-style factors, are most likely related to these risk changes. This is illustrated by the fact that, within two or three generations, cancer rates among Japanese migrants to the USA increase from those common in Japan to those prevalent in the USA (10). The same phenomenon has been reported for Polish immigrants to the USA (11). Although a number of reproductive variables have been associated with these changes, none could explain the differences. Mainly dietary factors are believed to be responsible for these risk changes (12).

Standard of living. It has been suggested that factors reflected by the standard of living might act independently from factors related to the nutritional status, for which the body-mass-index is an operational factor (13).

Familial clustering. Both genetic and hormonal factors have been related to familial risk for cancer but a recent study did not yield a significant effect of family history on Prol concentration in either generation two or three (14). Another study showed genetic factors to be involved in the case of a family history of BC: a specific locus was transmitted in some families with an extremely high frequency of BC through an unaffected father (15).

Inverse association with parity. The inverse association with parity has long been known for BC. It has been observed that this association is primarily the result of an positive association between BC risk and a woman's age when she has her first child (16). The sex of the first-born has been considered a risk factor for BC in case of a positive family history: androgens secreted by the boy-fetus would lower the risk (17).

Dietary factors. It has been suggested that differences in cancer incidence and mortality may be related to dietary habits. However, this is hard to prove based unambiguously on epidemiological evidence alone, because many other environmental differences occur at the same time. As will be discussed later examination of epidemiological data has revealed positive correlations between BC rates and intake of dietary fat, animal protein and simple sugars, but an inverse correlation with complex carbohydrates (18,19). A recent prospective study with BC patients demonstrated dietary fat intake to be inversely associated with survival time after treatment of the disease (20). It was estimated that the relative risk of death increased 1.4 times for every kg fat consumed monthly. In fact, the strongest evidence for the involvement of dietary factors in carcinogenesis comes from studies with experimental

animals. In general they provide support for epidemiological associations and can be used to elucidate mechanisms by which dietary fat may affect breast carcinogenesis.

Obesity. Prospective studies on post-menopausal women indicated that both body weight and height affect BC risk (21,22). A concept was developed that BC is one disease with two causes, one in pre-menopausal women and the other after the onset of the menopause (21). Further investigations on BC patients showed that only beyond the menopause overweight tends to be a risk factor, and that the relationship of BC with diabetes and hypertension is explicable by their associations with obesity (21). A recent Italian case control study on BC supported earlier results indicating a higher risk of BC in obese woman, and the trend of increasing risk with increasing body mass index being confined to post-menopausal women (23).

Ovarian activity. A cluster of associations points to a critical role of ovarian activity in the genesis of BC. The most direct evidence is the markedly reduced risk after artificial menopause induced by removing ovarian function either by surgical removal or radiation. Ovarian involvement is further evidenced by the association of increased risk with both early menarche and late natural menopause. However, there is conflicting evidence, if these characteristics differ in prediction of BC diagnosed before and after the menopause (24).

Fecal steroid metabolites. Women with BC as well as women with fibrocystic disease, incorrectly considered to be a high-risk group for the development of BC (25,26), have a higher excretion of total fecal steroids (in mg/g dry weight) than healthy women. Increases in both total neutral steroids and total bile acids contribute to this high fecal steroid level, which was not found to be related to obesity (27).

Additional indications with regard to BC include benign breast disease, other malignancies and ionizing radiation (7,8).

3. ANIMAL MODELS FOR THE STUDY OF MAMMARY CARCINOGENESIS.

Most of the early laboratory investigations of BC were carried out in mice. However, there are serious disadvantages of the mouse model. The tumors arise slowly and in most strains they are hormone-independent once the tumors reach palpable size (28). Around 1960 it was found that a single large but tolerable

dose of potent carcinogens evoked selectively and rapidly hormone-dependent mammary adenocarcinomas in the rat. Rat models for studying the process of mammary carcinogenesis in both outbred Sprague Dawley and inbred (Fischer F344, COP, BuF, Lew) strains are now reasonably well standardized and widely used. It should be noted that spontaneous mammary carcinomas occur much less frequent in rats than in mice, although benign fibro-adenomas are common in females of many strains. Further development and analysis of the models are needed to resolve conflicts in experimental data and to be able to extrapolate findings to the human species.

In general it is accepted that carcinogenesis is at least a two step process: initiation followed by promotion. Initiating compounds are found among chemical and physical agents such as some polycyclic hydrocarbons (MCA, DMBA), N-nitroso compounds (NMU) and ionizing radiation (29). Promotors are found among chemical agents, immunological and nutritional factors, and hormones. In animal models experimentation with either chemically induced, or radiation-induced or transplantable tumors can be used. For research on nutrition and mammary cancer animal experiments are mostly performed with chemically induced tumors. The chemicals most commonly used are NMU, DMBA and 2-acetylaminofluorene (AAF). NMU does not require metabolic activation to be carcinogenic (30), while DMBA and AAF are known to require metabolic conversion into their ultimate carcinogenic form (31).

3.1 The DMBA model.

Huggins et al. found that a single feeding of 7,12-DMBA, first synthesized in 1938 (32), to female SD rats of 50 days old rapidly elicited mammary tumors (33,34). It was recognized that the optimal dose for a single meal is 20 mg of 7,12-DMBA, dissolved in 1 ml of sesame oil and administered by gastric intubation. It was shown that the DMBA-induced mammary tumors were hormone-responsive, just as NMU-induced tumors (34). Low levels of the carcinogen however, evoked mostly fibroadenomas rather than carcinomas (35). Actually the mammary gland tumors induced by a single administration of DMBA comprise a spectrum of morphology from benign, typical fibroadenomas and adenomas to papillomas with hyperplastic, atypical or dysplastic epithelium and significant stromal and myoepithelial components, to tumors that are architecturally and cytologically malignant and invade adjacent tissue (36).

Metastases from even the most anaplastic tumors are rare (36). The characteristics of this "Huggins" tumor have been reviewed recently by Welsch (37). Factors that have a great impact on tumor incidence, tumor multiplicity and time to first tumor appearance (latency) include the age of the animal at exposure to the carcinogen, dose of the carcinogen and route of administration, the reproductive history, the endocrine status and dietary composition (36).

It has been demonstrated that there is a definite genetic component in the susceptibility to DMBA- and NMU-induced mammary adenocarcinogenesis (38). Recently it has been reported that the female COP rat is essentially completely resistant to all attempts to induce mammary adenocarcinomas by either DMBA or NMU exposure, due to the Mendelian inheritance of a dominant, autosomal genetic allele (38). In addition, although dietary fat does not appear to influence uptake and clearance of DMBA by mammary tissue (39), it is still conceivable that fat alters metabolic activity of the liver (40,41).

3.2 The NMU model.

The NMU-induced mammary tumor of the rat, first characterized by Gullino et al. (42) in female SD, BUF/N and F344 rats, is often used as a model tumor for human mammary carcinoma (43). Gullino et al. also have provided evidence that the NMU-induced mammary tumor readily metastasizes to spleen and bone marrow (42), but this observation has not been confirmed by others (44). The histological features of these tumors have been described (44). It has been reported that the percentage of malignant tumors increases, and the histological patterns may alter with increasing number of NMU doses (45). Susceptibility to carcinogenesis by NMU is strongly age-dependent, with a higher response if the exposure to the carcinogen has been at an early age, i.e. 50-55 days and a much lower response when NMU is given at older ages (90 days or more; 46,47). Tumor response seems also to be higher and the time to first tumor appearance shorter if the carcinogen is applied at proestrus or estrus than at diestrus (48,49). The endocrine responsiveness of the tumor has been well established. Most of the NMU-induced tumors are hormone-dependent: the tumors decrease after ovariectomy (50). Hypophysectomy of rats bearing NMU-induced mammary tumors causes rapid tumor regression (51). Additional administration of Prol or estrogen has been reported to stabilize tumor growth, and administration of Prol plus growth hormone (GH) or Prol plus estrogen to

hypophysectomized rats bearing regressing tumors results in growth reactivation, as reported recently (37). Progesterone may have an inhibitory effect on tumor development (52). One report has indicated that pregnancy and lactation after NMU treatment suppressed the development of mammary cancer (53). NMU-induced tumors contain receptors for E_2 (54,55,56), Prog (54,55), glucocorticoids (57) and Prol (55).

Animal studies suggest that the NMU-induced mammary tumor resembles the human mammary adenocarcinoma better than MCA- or DMBA-induced tumors do (42). Fischer rats (F344) appear to be most susceptible to the modulating effects of dietary fat on NMU-induced mammary tumors (58).

3.3 Other models?

In addition to the NMU and DMBA models, 'spontaneous' feline, canine and human BC share histological types and have similar biological behavior. As a high percentage of feline and canine mammary tumors is also estrogen dependent, both cat and dog may be useful models for hormonal studies and for the development of endocrine therapy for human BC (59). However, it should be noted that dogs have not been widely used as animal models in breast cancer research for a number of reasons, including their relatively long life span, the lack of inbred animals and a.o. the considerable expenses involved in their husbandry. Some of these reasons are also relevant to the feline model. At this time very little is known concerning the role of hormones in feline and canine mammary neoplasms. In addition, reports of mammary neoplasms in nonhuman primates are extremely scarce, as are reports on mammary neoplasms in rabbits and in guiney pigs.

3.4 Origin of chemically induced mammary tumors.

Mammary glands originate from epidermal epithelium, forming ducts that penetrate into the fat pad, where they ramify. In the young virgin rat (3 weeks of age) the ducts are made up with two layers of epithelial cells, an outer myoepithelial layer and an inner luminal lining layer. A differentiation between luminal and myoepithelial cells appears around the time of birth. The ducts are terminated by semisolid end-buds. The myoepithelial layer contains several cell types, of which two are pluripotent. It contains the stem cells

for mammary development, which are also present in the end-bud, and a precursor of ductules and alveoli (60). Studies of intraductal NMU-induced carcinomas revealed that the tumors contain both basal and luminal cells (61). This led to the conclusion that they must originate from multipotent cells, probably the stem cells, present in the end-buds. The origin of tumors induced by either NMU or DMBA has been studied by determining the earliest lesions after carcinogen administration and by determining the fate of these lesions. The early lesions include hyperplasias and formation of hyperplastic alveolar nodules (in mice) or adenomas (in rats; 62,63). The main alterations have been found in the end-buds. The change of susceptibility of rats to cancer induction with age is associated with changes in the end-buds (63,64), as well as with hormonal stimulation (64). It appeared that carcinogens are most effective when terminal end-buds are most numerous and their cells most actively involved in replication. The high rate of DNA replication makes the terminal end-buds in rats most vulnerable and prone to carcinogenesis at that time (65).

4. HORMONES IN THE ETIOLOGY OF BREAST CANCER.

For several decades research has been conducted on etiologic hypotheses for BC. Epidemiologic, clinical and animal studies claimed a role of hormones, mainly estrogens, in the pathogenesis of BC. Little is known to date about the etiology of mammary cancer, and the underlying biological mechanisms are not clearly understood (66). Etiologic evidence is generally discussed in terms of several major types of epidemiologic evidence such as geographic correlations, migrant studies, low-risk and high-risk populations, reproductive and endocrine status, and dietary modification trials. Nutritional factors such as specific excesses or deficiencies of nutrients apparently are able to modify mammary carcinogenesis in animals in a variety of ways (see section 5; 67,68). Furthermore it is suggested that factors of genetic, viral or hormonal origin play a more or less important role (69). Many human breast tumors are hormone-sensitive: they undergo striking regression when deprived of supporting hormone by removal of ovaries, adrenals, or pituitary, or by altering the hormonal status through administration of androgens, large doses of estrogens or anti-estrogens (70). This regression however, is temporary as recurrence often occurs later. The hormones most likely involved in these pathogenic cellular

changes are estrogens and Prog, but androgens, Prol, thyroid hormone, GH and insulin may also play a role. Estrogens induce proliferation of human breast tissue and may therefore be expected to increase risk of BC by stimulating the growth of stem and intermediate cells.

Seasonality has been associated with a number of hormone-related characteristics, e.g. duration of menstrual cycle, onset of menarche and birth rate. Seasonality in the occurrence of BC has also been suggested. Peaks occur in spring and troughs in autumn. It has been suggested that the pattern is of endogenous, probably hormonal, nature although environmental factors cannot be excluded (71).

4.1 Hormonal factors and the breast.

More than perhaps any other organ, the breast is under multisystem endocrine control (72). This control begins during intra-uterine life, when estrogen and testosterone exhibit separate effects on breast differentiation. From puberty, cyclic hormonal secretion results in a dynamic and changing pattern which is continued through adulthood until menopause. The responsiveness of breast tissue to hormones is to some extent genetically controlled. The pituitary makes breast tissue sensitive to estrogen, and there is evidence that ACTH stimulates the adrenal gland, which enhances the ovarian hormonal effect (72). Cortisol secretion by the adrenal cortex is necessary for the breast to function normally. Lack of cortisol is associated with atrophy of breast tissue (73). Prog causes alveolar cell growth in the estrogen-primed breast, but also cellular differentiation. The effect of testosterone on the breast is predominantly sex-related. Testosterone inhibits breast growth and lactation in women. Thyroid hormone enhances breast development, probably by potentiating the effect of ovarian hormones (73). Insulin affects the breast by enhancing the metabolic function of ductal tissue and by improving lactation (73). During pregnancy, placental hormones influence the breast resulting primarily in maximum development for lactation.

The physiological and metabolic state of neoplasms of the mammary gland is also subject to regulation by hormones. Many observations in both human and animal studies during the past decades point a dominant role of hormonal factors in mammary carcinogenesis. However, it is not clear whether one hormone, a particular class of hormones or an imbalance between (groups) of hormones is involved. The risk of the disease is thought to be fundamentally determined by

the hormones of the pituitary gonadal axis (74). The hormones that appear to be particularly relevant to mammary carcinogenesis in rodents are Prol, GH, estrogen, glucocorticoids, insulin and thyroxin (75). However, it appeared that once mammary tumors become advanced, they lose their hormone responsiveness and grow progressively in animals deprived of their endocrine function. In addition, mammary tumors do not develop in mice or rats that are either hypohysectomized or ovariectomized-adrenalectomized early in life.

4.2 Prolactin and breast cancer.

The remission observed in BC patients after hypophysectomy has been thought to be a result of the removal of pituitary Prol. Measurements of plasma Prol levels in women with BC have yielded contradictory findings. Sometimes the mean Prol level of patients with metastatic BC was higher than of controls, while in other studies there were no differences (76). Epidemiological findings indicate that pregnancy at an early age, which involves a large increase of blood Prol concentration, results in a lifelong reduced risk of BC. In countries where prolonged lactation (prolongued high blood Prol levels) is common, the incidence of BC is low. However, creating a clear picture of Prol in relation to risk variables is not easy because of the multitude of factors that can influence blood levels of Prol. Blood Prol concentration is dependant on known risk factors such as parity and age but also on the time of the day at which blood is sampled. In pre-menopausal women the stage of the menstrual cycle may be another confounding factor (16). Prol levels in the follicular phase were lower than those at mid-cycle or during the luteal phase. A positive association between overweight and Prol levels may be explained by increased estrogen production due to adipocytic aromatase activity (16). Women with a history of oral contraceptive use had low Prol concentrations (16). These findings support the hypothesis that body weight, parity, and age may influence BC risk in part by affecting blood Prol level (16).

Nagasawa (77) has suggested that the rate of DNA synthesis (and thus the risk of initiating events taking place) of normal mammary glandular epithelium is largely dependent on the level of circulating Prol. Nevertheless, it is not very likely that Prol plays a critical role in human BC since pregnancy and lactation are the only times of massive Prol exposure whereas lactation and pregnancy actually decrease BC risk, as known from epidemiological data. Our

current understanding of the relationship between estrogens, Prol and the mammary gland indicates that E_2 regulates Prol secretion via the hypothalamo-hypophysial system and, besides acting directly on mammary epithelium, enhances the sensitivity of mammary tissue to Prol stimulation.

A role of Prol seems to be more pronounced in animal studies than in human studies. It is known from animal studies that estrogens act synergistically with Prol at low concentrations, and antagonistically at high concentrations (78,79). It has been suggested that the ratio of circulating Prol to circulating estrogens (E_2+E_1) may play a central role in affecting mammary tumor growth and that this ratio is subject to perturbations by extrinsic factors such as dietary fat (80). A positive correlation between the plasma levels of Prol and susceptibility to mammary carcinogenesis by carcinogenic hydrocarbons was observed in three strains of rats (81). A high Prol:estrogen ratio may enhance and a low ratio may inhibit mammary cell proliferation, and thereby influence susceptibility to mammary carcinogenesis (see earlier). Dietary fat may influence mammary carcinogenesis via alterations in the relative concentrations of circulating Prol and estrogen (82). Plasma Prol has been shown to be higher at pro-estrus-estrus in rats fed HF diets (82).

4.3 Estrogens and breast cancer.

Many scientists suppose that steroids play only a permissive role in BC (83, 84,85): steroids may act by allowing or facilitating the primary action of other agents, such as chemicals, viruses or other hormones, or by promoting cell proliferation, providing greater opportunity for carcinogens to initiate neoplastic changes and/or for promotion to occur. Another view is that their main role is enhancement or maintenance of tumor growth (86). There are observations indicating that estrogens act by stimulating the growth of mammary cells in a late stage in the process of neoplastic transformation (85). However, the breast appears to be less susceptible to the tumor promoting effects of estrogen than the endometrium, as larger amounts of estrogens are required to achieve an observable alteration in risk of malignancy (85).

It must be stressed that there are no definite indications that hormones per se are causative carcinogens in man. As to the permissive role of estrogens Korenman (24) developed the "estrogen-window hypothesis":

1. Human BC is induced by carcinogens in a susceptible mammary gland.

2. Unopposed estrogenic stimulation is the most favorable state for induction.
3. There is a long latency between induction of a tumor and its clinical expression.
4. The duration of the estrogen window determines risk.
5. Inducibility declines with establishment of normal ovulatory menses and becomes very low during pregnancy.

It has been proposed that the most conducive environment for tumor induction would be estrogenic stimulation in the absence of influences of progesterone and of the hormonal consequences of the latter half of pregnancy. During certain periods of normal reproductive life, there would be open windows for carcinogenesis. Abnormal conditions and environmental influences could alter the duration of these estrogen windows (24). Estrogen-progesterone imbalance in favor of the estrogens is considered to be an important factor in the development of mammary cancer, although estrogens are not directly mitogenic. Analysis of steroid hormone levels in plasma of BC patients and normal women did not reveal any consistent differences. However, in both benign and malignant breast tissue, sex steroid hormone concentrations (ng/g) in tissue are significantly higher than in plasma (ng/ml). Concentrations of DHEAS, E_1S, T, 5α-androstane-3α-17β-diol and Prog are lower in benign and malignant tissue than in normal breast tissue (87), indicating differences in local (in situ) steroid metabolism between normal and neoplastic mammary tissue.

Both liver and intestine play an important role in estrogen metabolism. Estrogen conjugates formed in the liver become hydrolysed to a considerable extent in the intestine, by bacterial and human types of β-glucuronidase activity which increases from the upper to the lower small intestine and is strongly influenced by pH. Several metabolic processes such as hydrolysis, reabsorption, reconjugation and unchanged resorption can take place in intestinal mucosal cells and therefore influence overall estrogen metabolism (88,89).

Part of the estrogen metabolites in feces are products of microbial metabolism. The significance of such processes is not completely understood; most likely they have biological importance in deglucuronidation of estrogens in the intestine (88). E_1 and E_2 levels in plasma of pre-menopausal Caucasians were shown to be 30-75% higher as compared with age-matched Oriental immigrants from Southeast Asia to Hawai, the lattter eating less fat and more fiber. Menopausal Caucasians had 3-fold higher levels of E_2. The Oriental

women excreted more than twice the amount of estrogen in their feces but significantly less in their urine (90).

Urinary estrogen profiles of women belonging to high-risk populations differ from those of women of low-risk groups (91). This has led to the "estriol hypothesis": mammary tumor risk is inversely related to the ratio between E_3 and $(E_1+E_2+E_3)$. Comparison of urinary estrogen profiles (92,93) support this hypothesis, but comparison of plasma estrogens and Prol does not (74). Lemon et al. (94), postulated that E_3 may modulate the effect of more potent estrogens by inhibiting the binding of these estrogens to specific estrogen receptor (ER) in the breast cell. However, this so-called "impeded estrogen hypothesis" theory does not meet much support any more. It has also been suggested that a metabolite, 2-OH-E_1, may prevent mammary carcinogenesis by binding to the cytoplasmic ER, but without estrogenic effect (95).

Summarizing, there is considerable evidence that estrogens play a role in BC. Besides a permissive role also a direct effect on the initiating events has been suggested (96). The involvement of 16α-hydroxylation is believed to be relevant in human and mice breast carcinogenesis (96). However, in rats there are no indications of an active role of 16α-hydroxylation, because the female rat seems to be devoid of the 16α-hydroxylase enzyme (97,98). 16α-Hydroxy-E_1 is mainly relevant to mouse tumors, which is supported by findings in human BC (99). It has been suggested recently that the amount of non-protein-bound, so-called biologically available estrogen might be the key factor in the etiology of BC (100). Anti-carcinogenic activity of E_3 has been demonstrated if this hormone was administered to 50-55 day old SD rats prior to treatment with DMBA (101). However, no explanation for possible mechanisms is available.

4.4 Other hormones related to breast cancer.

Post-menopausal vegetarian women have been found to excrete slightly less estrogens in their urine than carnivorous women and to have a higher SHBG, indicating that less unbound estrogen is available in the vegetarians (102). Very recently it was reported that plasma SHBG levels were significantly lower in women with breast cancer than in either the control population or matched controls (103). Hormonal studies on relatives of women with BC have yielded inconsistent results. Daughters of patients have been reported to have increased levels of plasma Prog and urinary pregnanediol (104). In a large

prospective study it was shown that women who excrete low levels of etiocholanolone and androsterone have elevated risks of subsequent BC (105).

Although glucocorticoids, thyroid hormone and GH influence the development of the mammary gland, the importance of these hormones for mammary tumor growth in rodents is less clarified (37). The meaning of glucocorticoids, insulin and thyroid hormones in rats has been discussed recently (106).

4.5 Conclusion.

Hormones most likely affect risk of BC not directly by causing damage to cellular DNA as do ionizing radiation and chemical carcinogens, but by altering the kinetics of susceptible stem cells of the mammary epithelium or of stem cells that have already undergone some degree of malignant transformation. The risk of initial transformation is enhanced as a result of stimulated stem cell proliferation on the one hand and cyclic exposure to endogenous steroids on the other hand. Maybe the risk of BC is greatest between menarche and the birth of the first child. Exposure to initial transformation may be caused by environmental factors, including dietary factors. Further proliferation of primed stem cells may depend on different (un)specific shifts in the endocrine milieu, including Prol, Prog and androgens as well as disturbances in their metabolic pathways, or may even be independent of hormones at a later stage.

5. STEROID HORMONE RECEPTORS.

Hormone responsiveness of endocrine target tissues has frequently been assessed by qualitative or quantitative measurement of receptors for the hormone involved. The current understanding is that steroid hormones enter their target cells, where they bind to specific binding proteins with high affinity, called receptors. The steroid-receptor complex is then activated. The activated complex is loosely bound in the cytosol and tightly bound in the nucleus where it binds to chromatin or DNA, or both. The activation step is energy-dependent and can be accomplished by binding the steroid at 0°C and raising the temperature to 25-37°C (107). The activation step involves a conformational change, exposing new lysine residues in the receptor (108). From in vitro and in vivo studies the possibilities have been raised that pyridoxal

5'-phosphate, and hence vitamin B_6, can act as modulator of steroid activity by binding to the ε-lysine groups of the specific steroid receptor molecules thereby inhibiting activation or nuclear translocation and binding to chromatin, or both. Although hormones have many effects on their target tissue, one of their principal effects related to carcinogenesis appears to be their growth promoting action on these cells (109). Typical of *in vivo* hormonal studies has been the basic finding that target tissues regress after removal of specific endocrine glands (such as ovaries, adrenals, pituitary) and that growth can be restored and even stimulated in these regressed tissues by supplying the host with specific hormones from the endocrine glands concerned.

To achieve a better understanding of the role steroids may play in carcinogenesis, abnormalities in steroid metabolism in cancer patients should be looked for, which might serve as "markers" and may thus be of value for population screening. In the same approach a hormonal environment may be defined that is favorable for the development of cancer. Steroid metabolism is known to be affected by a variety of factors including diet and nutritional status. For example, nutritional status is related to the adrenocortical function, in particular to corticosteroids (84). Blood levels may be indicative of hormone production and of tissue exposure. Measurement of urinary steroid concentration covers overall production and metabolism, but may be difficult to interpret. The amount of steroids in specific tissue is probably the most reliable indication of tissue exposure to specific steroids. The best indication of tissue sensitivity to steroids is the specific steroid receptor present in the tissue. It is very likely that most, if not all, effects of estrogens and androgens are mediated by their binding to these specific receptor proteins.

5.1 Estrogen receptors.

Estrogen receptor (ER) must be present for estrogen to influence the biological activity and growth rate of breast tissue cells (110). Although estrogen binding proteins may exist in normal breast tissue to regulate breast development during puberty and pregnancy, they are usually not present at biochemically measurable levels (111). However, the use of monoclonal antibodies may show their presence. Cellular receptor proteins that can bind estrogens have been identified in a variety of tissues including uterus,

vagina, corpus luteum, mammary gland, mammary tumor tissue, both in women and in experimental animals, and also in pituitary and hypothalamus of both sexes (112,113). A cytoplasmic ER is a macromolecule that binds E_2 with high affinity ($Ka = 2.10^{10} - 1.10^{9} / M^{-1}$) and specificity but with limited capacity and a limited number of receptor molecules per cell ($3.10^{3} - 4.10^{4}$). Estrogens enter their target cells and initiate a series of steps to exert their effect at the level of gene transcription, resulting in specific protein synthesis (114). Initial cell penetration is by passive diffusion. The ER complex has a sedimentation coefficient of 4S. At physiological temperatures the complex is activated, which is associated with an increase of its sedimentation value to 5S (115,116). The steroid-receptor complex binds in the cell nucleus to chromatin "acceptor" sites and in some way as yet unknown modulates messenger RNA synthesis which appears to be characteristically restricted in hormone-dependent tissues (117,118) and which is followed by protein synthesis and cell growth, DNA synthesis and proliferation (119). It has been hypothesized that activation of the gene coding for receptor protein and expression of ER in breast tumor tissue is part of the process of neoplastic transformation (120). The final effect of a hormonal stimulus is believed to depend on the number of hormone receptor complexes bound to the target cell chromatin, hence a response would directly depend on the number of receptor sites available for hormone binding. The extent to which receptor status reflects the natural history of the disease process prior to exposure or host influences remains to be elucidated. Recently, it has been reported that ER may be residing in the nucleus rather than in the cytoplasm and suggesting that cytoplasmic ER may be a procedural artefact (121,122).

5.2 Estrogen and progesterone receptors, and mammary cancer.

In 1972, Jensen (123) observed that the ER is responsible for the uptake of estrogen by BC tissue. The presence (ER-positive, ER+) or absence (ER-negative, ER-) of receptor protein and the concentration (estimated number of binding sites) in malignant breast tissue are established prognostic factors in the clinical course of BC. The measurement of the ER in BC tissue may predict the degree to which a BC patient with an intact receptor mechanism may benefit from estrogen therapy and can have a more favorable prognosis (124, 125). It is as yet not clearly understood which role ER plays in mammary carcinogenesis per se.

The presence of ER in human mammary tumors has been studied extensively. Approximately 60% of primary mammary tumors in women contain detectable amounts of ER (126,127). However, only 25 to 30% of the cases of human BC is responsive to endocrine manipulation (128). The failure of one-third of the patients with receptor-positive cancers to respond to endocrine therapy is probably due in some cases to tumor heterogeneity and in other cases to the fact that receptor does not function (117). The significance of the presence of ER in mammary tumors for predicting the response of the tumor to endocrine therapy has been widely studied. In a collaborative study of eight cancer institutes 54% of ER+ mammary tumors regressed after endocrine therapy, whereas only 5% of ER- tumors showed a regression (126). The response to hormonal treatment was significantly higher in patients with ER+ primary cancers, and the likelihood of response was proportional to the measured receptor concentration (129). Receptor status seems to vary according to age, menstrual status, parity, race and other risk variables that also affect prognosis (118).

The pattern of distribution of distant metastases has been observed to be associated with ER status of the primary tumor: ER+ tumors tend to recur in bone, ER- tumors show affinity for viscera (130,131,132). It has been suggested that the ER determination would be more reliable in conjunction with the determination of c-AMP-binding protein (133). In contrast to the cytoplasmic ER the nuclear ER was highest in the central parts of mammary carcinomas (134). These differences in ER content were found in both pre- and postmenopausal women. Steroid receptors have also been demonstrated in male BC (135). It has been reported that high plasma estrogen levels are strongly associated with low cytosol ER values, while low (post-menopausal) estrogen levels can be associated either with low or with high ER values (136). E_1S in tumor tissue can be converted into E_2 and might influence receptor levels (137). ER+ tumors contained significantly more E_2 than ER- tumors.

The correlation between the presence of ER and the result of endocrine therapy is beyond doubt now, but the acting mechanisms are still unknown. Several studies confirm that patients with ER+ primary tumors have a better prognosis than patients with ER- primary tumors (138,139). Determination of the progesterone receptor (PR) has been reported to give similar or even more accurate information. However, contradictory data are available on the relation between ER and PR status and the disease-free interval in BC. There are some explanations for these conflicting results: ER assays have not always

been performed from primary tumors, specimens from metastatic sites are retrospective in nature, and discordance in receptor status in sequential specimens has been reported to be 23% for ER and 30% for PR assays. In addition, in some studies a large part of the patients had received some form of chemotherapeutic or endocrine treatment. Some reports lack information on these two important factors. Since the efficacy of adjuvant endocrine treatment has been shown to be related to the presence of ER in the tumor tissue, this kind of treatment certainly complicates the evaluation of the relationship between receptor status and the disease-free interval. Several other factors may also account for disparities between results, including different cut-off values for defining ER+ tumors and variations in detection limits associated with different techniques for estrogen estimation. Generally, in BC evaluation the PR is taken into account as well (140-144). The highest frequencies of receptor positivity are observed in patients with small and well differentiated tumors (139). There is some support for the assumption that estrogens induce the PR in human BC, and that this may be mediated by a mechanism involving nuclear receptors (145). An explanation has been proposed (146), that suggests that determining PR content in the tumor, which in normal reproductive tissues is known to depend on estrogenic stimulation, might serve to identify tumors with functioning receptor. Under normal circumstances ER and PR are interdependent in the same cellular system. During neoplastic transformation the capability of estrogen sensitive tissues to synthesize PR proteins may be altered. BC cells containing substantial amounts of both ER and PR show a significantly higher response rate to hormonal therapy, so that measuring both receptors can increase the accuracy of the predictive assay. However, some cancers with high levels of both receptors still do not benefit from hormonal therapy, while many containing only ER do respond. At the present state of knowledge, PR assays can complement but not replace ER assays. PR is rarely found in ER-metastatic breast tumors but is present in approximately 59% of ER+ metastatic tumors, specially in those with high concentrations of ER (147). From a theoretical point of view, however, it is not surprising that a positive correlation between Prol receptor and ER and PR has been reported recently, since many studies on both experimental mammary tumors and normal tissues have shown that Prol, E_2 and Prog as well as their receptors are interrelated (148,149). So far, clinical studies have yielded diverging conclusions regarding associations between receptor status and prognosis of the disease and/or the outcome of endocrine therapy. The

inconsistencies are diverse in character: non-uniform sampling of tumor tissue, differences in receptor assay systems used, and differences in the composition of the patient populations.

5.3 Estrogen receptors in animals.

In DMBA-induced rat mammary tumors ERs have been demonstrated (150). These tumors are estrogen sensitive as they regress after ovariectomy (151). NMU-induced tumors show similar estrogen sensitive properties (54). Experiments with NMU-induced tumors as compared with DMBA-induced tumors suggest that NMU tumors are more responsive to endocrine manipulation coincident with NMU injection, but that hormonal treatment of established tumors is somewhat less effective (55). It has been demonstrated that cytoplasmic and nuclear ER levels are correlated with the concentration of circulating estrogen for rat uterine tissue (152). Specific modification of ER in rat uterus (153) by pyridoxal 5'-phosphate may point to implication of vitamin B_6 in the physiological regulation of estrogen action. Further findings on pyridoxal phosphate and steroid action have been mentioned earlier (see also ref. 154). Ionizing radiation of mammary tumors (20 Gy) reduced the concentration of both ER and PR in the rat DMBA model (155). ER content appeared to be changing during the different stages of the estrus cycle in rat uterus (156).

5.4 Androgen receptors.

Cellular receptor proteins that can bind androgens have been found in a variety of animal and human tissues such as prostate, seminal vesicles, testes, epididymides, kidney, sebaceous glands, hair follicles, various areas of the brain, pituitary, muscular tissue, bone marrow, liver, lung, heart and pancreas. In addition they have also been demonstrated in the ovary, uterus, mammary tumor tissue [DMBA-induced, Shionogi S-115 mouse cell line, in human tissues and cell lines (112,157)]. Although T has been shown to cause regression of the estrogen dependent DMBA-induced rat mammary tumor, this was not established in transplantable tumors (157). The significance of AR in breast tumors is uncertain. Nevertheless, the simultaneous presence of AR along with ER and PR might increase the likelyhood of response to endocrine therapy over that associated with tumors possessing ER or PR alone. Little is known about the significance of these findings for BC.

5.5 Nutrition and hormone receptors.

ER levels in mammary tumors are reduced in rats fed 0.5% fat (corn oil) compared with rats fed 5 or 20% fat (158). In the rat DMBA model no nutritional influences on PR concentrations have been observed. In a subsequent study it was found that rats fed a LF or an HF diet did not express altered induction of PR in normal uteri (159). The quantity of Prol receptors in DMBA-induced mammary carcinomas in SD rats was not changed by either HF (20%, corn oil) or moderate (4.5%) fat. However, in NMU-induced tumors an increased amount of Prol receptors was observed in animals fed a HF (20%, corn oil) diet as compared with rats on LF (0.5%, corn oil) (160). One may speculate that animals fed low to marginal or perhaps deficient levels of dietary fat and concomitantly deficient levels of EFAs demonstrate reduced hormone receptor levels. This might be a subtle influence of diet on the target tissue. On the other hand, it has been reported that a HF diet enhances the development of DMBA-induced mammary tumors in ovariectomized rats, indicating that the effect is independent of continuous production of estrogen by the ovaries (161). No marked changes in cytosol ER content have been observed in rat uterus during protein malnutrition (162).

6. DIETARY FACTORS IN THE ETIOLOGY OF BREAST CANCER.

Dietary factors are postulated to be important in the etiology of BC. The geographic and migration epidemiology of BC are very similar to that of prostate cancer and colon cancer (163,164) and indicate that these cancers seem to be associated with similar dietary factors (165). A 10-year prospective study of about 150.000 women in Japan aged 40 years showed that BC risk was 8.5 times higher in women of high socioeconomic strata eating meat daily than in women of low strata who did not eat meat daily (76). A high positive correlation was found between per capita fat intake and adjusted death rates owing to BC in different districts of Japan. It was estimated that the BC death rate will rise to the US level when Japanese dietary fat intake is on a level with the USA. Pork and animal fat contributed most to fat intake (76). A nutritional hypothesis finds support in experimental studies in rats and mice, in which dietary factors have been shown to be able to influence mammary carcinogenesis (166-170).

Cancer is considered to originate from a mutation in a single cell due to a carcinogen-DNA interaction. This primary process is referred to as initiation. Initiation is a rapid process and considered essentially irreversible. The subsequent proliferation of the damaged cells during a much longer period of time is referred to as promotional stage (171). Diet may influence tumor initiation by the presence of carcinogens in food. These include procarcinogens, naturally occurring substances, compounds formed during cooking or processing, preservatives, coloring or flavoring substances and pesticide residues (172,173). Additionally, the formation, activation or inactivation of carcinogens by the gut flora (172,174) may be important. Some compounds may act by altering enzyme systems which either detoxify chemical carcinogens or convert them into ultimate carcinogens. There is increasing evidence of a role of dietary factors in the promotional stage of mammary carcinogenesis (5,175-177). These may include the same or similar substances listed above for initiation. On the other hand, if dietary factors exert a modifying effect on breast carcinogenesis in animals, nutrition might also have preventive and perhaps therapeutic possibilities in man. The lower recurrence rate in ovariectomized animals on a LF versus a HF diet makes a reasonable case for prevention of recurrence in BC patients through dietary manipulation (178). A lower daily intake of total fat may be beneficial (179).

6.1 Dietary factors and human breast cancer.

A nutritional hypothesis of BC etiology is supported by studies on correlations between international BC mortality and the consumption of certain food products and food components. Positive correlations between age-adjusted mortality from BC and consumption have been reported for sugar (r=0.74-0.84), potatoes (r=0.38), meat (r=0.74-0.79), eggs (r=0.69-0.80), milk (r=0.73-0.79), fat and oils (r=0.80), total fat (r=0.63-0.89), animal protein (r=0.83), total protein (r=0.57) and energy (r=0.60). Negative correlations have been reported for cereals (r=-0.70- -0.77), vegetables (r=-0.31), citrus fruit (r=-0.43), pulses (r=-0.46), fish (r=-0.65) and selenium (r=-0.8). For total fruit contradictory values have been reported (r=0.44 and r=-0.10) (176,364). A positive correlation ($P<0.05$) between sugar and fat intake and BC mortality was found, in a study on annual per capita consumption of various macronutrients (180). Drasar and Irving found correlation coefficients of >0.75 between BC incidence and per capita intake of fat and protein (181). Total fat

consumption was strikingly related to BC incidence and mortality (182). A significantly increased risk was found for a higher consumption of milk and dairy products but the risk estimates were only slightly higher than those for meat consumption (23). However, this type of internationally correlations should be interpreted with care. On the other hand, the attractiveness of the correlation between geographic factors and dietary fat is increased by the fact that biochemical mechanisms may be assumed such as conversion from androstenedione into estrone in body fat depots.

The possible combinations between other dietary factors and human BC are too complex to discuss in detail here. For factors such as carotene, vitamins A, C and E and selenium reference is made to recent publications (183-186).

6.2 Total caloric intake.

Only a few epidemiological studies have examined the effect of caloric intake per se on the risk of cancer. From these studies mainly indirect evidence can be derived, based on associations between body weight or obesity and BC risk (183). Worldwide correlation studies have directly associated BC incidence and mortality with caloric intake, which in turn is correlated with intake of total fat, total protein and animal protein (175). Case control studies have pointed to stronger associations with dietary fat consumption than with total energy intake (187). As caloric intake and obesity are generally correlated, the effects of caloric intake can not be separated from that of body weight (21).

A number of studies in animals support a relation between excessive energy intake and cancer incidence in general as well as a non-specific shortening of life span (184,188). Caloric restriction, whether early or late in life, appears to decrease the incidence of tumors at multiple sites (189-193). The risk of a rat developing a spontaneous tumor has been shown to be directly proportional to caloric intake and growth rate early in life (189,194). Further studies on the role of caloric intake revealed that inhibition of tumor growth depends on the extent of food restriction, the type of tumor, and the dietary composition. It was shown that both the degree and the timing of caloric restriction are important determinants for the suppression of mammary carcinogenesis in rats. A 20% reduction in food intake over a 2-year period significantly inhibits the development of spontaneous mammary tumors in both mice and rats (195). It was demonstrated that rats fed a HF diet at 40% less

calories than LF controls exhibited significantly fewer tumors despite the consumption of more than twice as much fat in grams by the rats fed the restricted diet (193), suggesting that caloric intake per se is a more stringent determinant of tumor growth than fat intake (196). More recent studies indicate that tumor appearance does not depend on the fat content of the diet per se but rather on a complex interaction involving energy intake, energy retention and body size (197,198). Additional data demonstrate that the tumor promoting effect of dietary fat can be more than offset by a reduction in total caloric intake and that the promoting effect of fat may be due to its greater caloric density (199).

The mechanism of action of the energy effect is not fully understood. Because energy restriction induces a wide range of physiological changes, particularly in the endocrine and immune system, it is likely that several mechanisms mediate the inhibitory effects of underfeeding. It has been suggested that the long-term inhibitory effects of energy restriction on the formation of DMBA-induced mammary tumors in rats are largely the result of a kind of hormonal deficiency state at the time of tumor initiation and that this could be counteracted by elevation of blood estrogen and Prol levels (200,201).

6.3 Effects of macronutrients in animal studies.

6.3.1 Fat.

Laboratory evidence supporting the role of dietary fat in mammary carcinogenesis has been claimed first by Tannenbaum (202). In 1942 he reported a six times higher incidence of mammary carcinomas in mice fed a diet containing 12% fat (hydrogenated cotton seed oil) than in control mice at a LF diet. Numerous studies have confirmed and extended the observation of enhanced mammary carcinogenesis by increased consumption of dietary fat since (168, 203,204). This effect of dietary fat has been seen now in an impressive array of experimental rodent mammary tumor systems including spontaneous, carcinogen-induced, X-ray-induced, synthetic hormone-induced (DES), benign and malignant tumors, transplantable tumors, and in obese and non-obese animals. In general, the fat effect is characterized by increased mammary tumor incidence and/or a reduced latency period. In most studies isocaloric diets were

used, thus excluding increased caloric consumption as the one and only explanation of this effect of fat. The effect seems to be independent of caloric intake and has been demonstrated with spontaneous tumors as well as with tumors induced by a variety of carcinogens (18,29,58). The type of dietary fat is important (205,206): diets rich in poly-unsaturated fatty acids (PUFA; 20% by weight or 40% by energy; 206-208) are more effective in the promotion of mammary tumors than are diets rich in saturated fatty acids (SAFA; 208,209). Changes in the levels of PUFA affect the mitotic rate in mammary tissue (210), and the proliferation rate of tumor cells could be affected as well. Further studies have revealed that a minimal amount of dietary PUFA is required for a HF diet to enhance mammary carcinogenesis (207-209). Studies in mice fed corn oil, containing over 50% of linoleic acid (LA) have pointed out LA as the most crucial fatty acid responsible for this tumor-enhancing effect (211). Further support has been found in studies testing several oils and fats in the C3H/St mouse and associating dietary fatty acid levels in the diet with spontaneous mammary tumors (212). Increased tumor incidence and decreased time to first tumor appearance were observed with increasing levels of LA (18:2) at the expense of other fatty acids. Out of four SAFAs (laurate (12:0), myristate (14:0), palmitate (16:0) and stearate (18:0) only the last one showed a significant effect. It has been suggested that erucic acid (22:1) reduces tumor incidence, while oleic acid (18:1) has no significant effect (212). Results supportive for LA and linolenic acid but not for oleic acid as important modifiers of carcinogenesis have been found in studies with DMBA-induced mammary tumors in SD rats (207). Studies of Chan and Dao (213) support the HF diet theory for NMU-induced mammary carcinomas in Fischer, Long Evans and Sprague Dawley rats. Additional data in animals fed experimental diets since weaning show that an increase in fat intake enhances spontaneous mammary carcinogenesis in mice, but the magnitude of the increase depends on the type of fat. The sum of total energy derived from oleic acid and LA correlated positively (r=0.95) with mammary tumor incidence (214). However, daily intake of LA alone shows a correlation with mammary tumor incidence (r=0.85) while oleate did not correlate (r=0.4; 180). Similar correlations were observed for tumor multiplicity. In vitro studies support a stimulating role for oleic acid and LA in both normal and neoplastic epithelial cells (215). Comparison of a fat containing 38% trans isomers with another fat containing a similar amount of cis isomers showed no significant difference in their ability to promote DMBA-induced mammary tumors (216). SAFAs, either medium or long chain, and cis

and trans monoenoic fatty acids appear to have no specific promoting effect (216).

The enhancing effect of fat seems to be primarily active in the promotion of carcinogenesis (211,217). Studies with chemical carcinogens demonstrate a higher tumor yield when HF diets are fed after carcinogen treatment then when they are fed before treatment. Enhancement of mammary carcinogenesis is still observed if the HF diets are fed as late as 20 weeks after carcinogen treatment (218). Mammary carcinogenesis is also inhibited by reduction of fat intake (219). Fish oils containing long chain PUFAs of the linolenate type (n-3) appear to reduce mammary tumorigenesis induced by NMU (220) when fed at high levels, while they tend to enhance mammary carcinogenesis induced by either DMBA (207,221) or NMU (220) when fed at low levels.

6.3.2 Carbohydrate.

Rats fed soluble simple carbohydrates such as glucose or sucrose have been shown to be slightly more prone to tumor induction by DMBA than rats fed complex starch (222) such as wheat, rice or patato starch. This difference was observed at both low and high levels of dietary fat. In another study rats are fed diets containing sucrose, lactose or corn starch as carbohydrates after initiation with DMBA (223). Tumor yields were similar in the groups fed sucrose and corn starch but significantly less in the animals fed lactose. Consumption of starch was associated with the highest number of palpable tumors. These data indicate that the type of dietary carbohydrate fed during the promotional phase of DMBA-induced mammary tumors may significantly affect tumor yield. The effects of starch versus sugar cannot be generalized. The mechanism by wich carbohydrates affect tumor promotion is unclear. Data on carbohydrate and mammary carcinogenesis are too limited to permit any firm conclusion.

6.3.3 Protein.

Literature on a relation between protein and cancer is limited. Studies on DMBA-induced mammary tumorigenesis indicate that dietary protein can influence chemically induced mammary carcinogenesis by altering the development of neuro-endocrine and reproductive functions in rats, resulting in increasing incidence of tumors with increasing level of casein in the diet (8%, 19,5% or

31%; 224). Some experiments suggests protein to affect the initiation phase of NMU-induced carcinogenesis and/or the subsequent growth and development of mammary tumors (225). A combined high-protein/high-fat diet is lilely to corroborate the positive correlation between dietary fat intake and BC risk in women (226). Some animal studies failed to provide strong evidence of a causative association between dietary protein and carcinogenesis (166,179). Other studies with DMBA-induced tumors have failed to demonstrate differences in response between rats fed diets with casein or soya protein (166). In SD rats a diet with 50% raw soy beans has been shown to inhibit X-ray induced mammary carcinogenesis, possibly due to protease inhibitors present in soy beans (227). Varying the protein content at the same fat content was shown to have a minor effect on spontaneous mammary carcinogenesis in mice (228). Low amounts of protein in a HF diet have been shown to be associated with the highest incidence of DMBA-induced mammary tumors in rats (229,230). Conversely, the lowest fat content and the highest amount of protein were associated with the lowest tumor incidence. An effect of fat content on tumor incidence was observed in this study, but the interactive effects of fat and protein were greater than those of either component alone. The amount of protein fed during the promotional stage of carcinogenesis had no apparent effect, but initiation of tumors seemed to occur more readily in rats fed a low-protein diet. It has been investigated how protein content and fat content interact in the DMBA rat model (231). No substantial interactions of protein and fat were observed with regard to tumor incidence (231,232). Further analysis revealed that, in addition to the response to dietary fat, carcinogenesis increased in rats with a high ad libitum food consumption. The protein content of the diet did not influence the effects of fat on carcinogenesis except for a significant fat/protein interaction on the size of the adenocarcinomas (231). Higher dietary protein intake during the initiation phase was associated with a significant reduction in tumor prevalence, wich was most striking between 8% and 16% of energy from protein (232).

6.4 Dietary fiber and seaweed.

Dietary fiber is a generic term for dietary compounds that are not metabolized by intestinal secretory enzymes. Dietary fiber encompasses a variety of substances of unique structure having specific physical characteristics and physiological functions. Several studies have pointed to a protective effect

of dietary fiber on colon cancer (233). Only recently, some indications towards an effect of dietary fiber on BC have been reported. As opposed to a positive correlation between dietary fat and BC, a negative correlation has been reported between dietary fiber and BC in a life-span study on spontaneous tumors in rats (234). In another study on rats with diets low or high in fiber (wheat bran) and/or low or high in fat (lard), it was found that females on the high-fiber diets develop less spontaneous tumors (both benign and malignant) of the mammary gland than those fed the low-fiber diet irrespective of fat content of the diet (235). There are no studies of effects of dietary fiber on chemically induced mammary cancer in experimental animals.

Dietary fiber may reduce risk of cancer by adsorbing steroids (236) or inactivating carcinogenic or co-carcinogenic compounds (237,238). Recently, it was reported that intake of dietary fiber, mainly grain fiber, can significantly affect estrogen metabolism as indicated by reduced estrogen levels in urine (239), thus suggesting estrogen metabolism to be a possible site of action for dietary fiber in mammary carcinogenesis. Interestingly, it was reported that the phyto-estrogen equol competes with the E_2 receptor complex for nuclear binding and fails to initiate the replenishment of ER effectively in the cytoplasm (240).

The tumor-inhibiting effect of edible seaweed, Laminaria angustata, on 7,12-DMBA-induced mammary carcinogenesis has been studied in rats (241). This seaweed seems to be able to delay tumor development in rats as well as to diminish tumor multiplicity. Other seaweeds are supposed to be more effective in the inhibition of mammary tumors. Laminaria religiosa and Porphyra tenera both significantly delay tumor appearance (242). However, the inhibiting mechanism of dietary seaweed has not yet been clarified. It has been suggested that they minimize the toxicity and carcinogenicity of DMBA. Orally given seaweeds may partly potentiate the defence mechanism and partly act like dietary fiber, which directly minimizes the activity of the carcinogen.

6.5 Vitamins, trace elements and antioxidants.

Vitamins and trace elements are two other groups of dietary ingredients associated with carcinogenesis (243). Much attention has been focused on a possibly protective role of vitamin A (244-247) and its synthetic analogues (248). Synthetic retinoids are effective in preventing BC in animals (249, 250). Excess of various forms of vitamin A inhibits mammary carcinogenesis in

rats, but not in mice (251). Retinoids in combination with hormonal manipulation (ovariectomy/antiestrogens) are much more effective in inhibiting mammary carcinogenesis than either treatment alone; this combination also inhibits mammary tumor recurrence after surgical removal of the first tumor. The mechanisms by which retinoids inhibit carcinogenesis are unknown. In the mammary gland, retinoids inhibit cell differentiation and proliferation, DNA synthesis, and RNA polymerase activity (252).

The addition of tocopherol to the diet has been shown to reduce the number of DMBA-induced mammary tumors in rats fed HF (coconut oil) diets, but not in animals fed HF (safflower oil) diets (253). Vitamin E was shown to have no effect or to inhibit mammary carcinogenesis to an extent depending upon the method of its administration, or linked to the level of dietary selenium (Se) or dietary fat (251). In some studies both low (30-50 mg/kg) doses (254,255) and a higher dose (2 g/kg) (256) of dietary vitamin E were ineffective in preventing or inhibiting DMBA-induced mammary carcinogenesis in the rat. However, in other studies it appeared that vitamin E was effective when fed with a HF diet (255) or in conjunction with Se supplements (254). Se appeared less effective when given in a vitamin E-deficient diet (257).

Data on trace elements have been summarized recently (258,259). Se fed in the diet or in drinking water as Se_2O_3 received most interest as a potential inhibitor of various chemically induced tumors in mice (260). Se depletion seems to have a stimulating effect on mammary tumor incidence upon DMBA treatment in rats fed a diet rich in PUFAs, in contrast to rats fed SAFAs (217). Se has been protective even when it had been administered well after the carcinogen (185). Se suppletion resulted in a decreased DMBA-induced tumor incidence, sometimes in combination with prolonged average latency (217). The mechanism of Se action has been explored only partially. It is known that Se is an essential co-factor in glutathione peroxidase which acts as a cellular antioxidant. Inhibition of mammary cancer by Se was greatest in rats fed a HF diet rich in PUFA (260), which normally increases intracellular peroxidation. In addition, Se may have a favorable effect on carcinogen metabolism and may enhance immune defence (184). Selenoproteins have been identified in tissues suggesting that Se may function in mammary tumor inhibition after binding to intracellular macromolecules.

A possible role of pyridoxal phosphate in carcinogenesis is conceivable (261), since pyridoxal 5'-phosphate is required for functioning of more than 60 enzymes, including amino acid decarboxylases, transaminases, racemases and

enzymes of tryptophan and cysteine metabolism. There is experimental evidence that under certain conditions very large amounts of vitamin B_6 may inhibit Prol secretion (262).

The roles of zinc, copper and manganese in immune function and in experimental oncogenesis have been discussed recently (263). In addition, there is some evidence that iodine deficiency may increase susceptibility to mammary carcinogenesis (264). Molybdenum may inhibit NMU-induced mammary carcinogenesis (265).

Some indications are available of the role of antioxidants as inhibitors of mammary cancer (266-268). In the DMBA model, BHT impedes the stimulation of the HF effect (268). Preliminary data indicate that BHT did not inhibit the HF-effect in the NMU-induced mammary tumor (267). These results suggests that BHT interferes with DMBA-activation. In other experiments propyl-gallate was found to inhibit carcinogenesis (269). One may assume that promoting effects of PUFA are only partly impeded by dietary antioxidants.

7. NUTRITION AND HORMONES.

As outlined above, mammary carcinogenesis is believed to be related to dietary and hormonal factors. It is therefore attractive to assume that diet affects BC through endocrine mechanisms.

7.1 Diet and prolactin.

The activity of the endocrine system and the activity of endocrine target tissues are influenced by dietary fat. In conjunction with studies linking hormones and dietary fat to enhanced mammary carcinogenesis it was hypothesized that dietary fat acts by increasing plasma levels of particular hormones, most likely Prol and estrogens.

7.1.1 Animal studies.

Some animal studies suggest that a high dietary amount of fat could be responsible for an enhanced mammary tumor incidence by stimulation of pituitary Prol secretion (18). The first report (82) claiming that female SD rats fed a HF diet (20% w/w) exhibit higher serum Prol levels during

proestrus-estrus than rats fed a LF diet (0,5% w/w) has been confirmed several times (58,270,271). It has been shown that in NMU-treated rats a HF diet results in higher absolute levels of Prol and a higher Prol over estrogen ratio than a LF diet (58). Feeding diets containing high or low levels of lard results in elevated plasma Prol levels in HF diet groups of male rats as compared to animals fed a LF diet (272). In contrast, several studies have failed to show any effect of dietary fat on serum levels of Prol in rats (5,207, 273-,274). When DMBA-treated rats fed either a HF or LF diet, were also given an anti-Prol drug, both groups showed an equally decreased number of tumors. These and other studies indicate that the enhancing effect of HF diets on mammary carcinogenesis may be mediated via hypothalamic or pituitary functions (82). This concept is consistent with the observation that fat influences primarily the promotional phase of carcinogenesis as has been reviewed elsewhere (275,276). Enhancement of Prol secretion in rats strikingly alters the fatty acid composition of the mammary gland, namely from growth inhibiting saturated fatty acids to growth promoting unsaturated fatty acids (215). There are experiments indicating that in ovariectomized rats a HF diet has a tumor enhancing effect even in the absence of normal periodic ovarian secretions (277). After an initial tumor regression after ovariectomy, the tumors regrew faster and in larger numbers in rats on a HF diet. A study on the combined effect of dietary protein and fat on Prol levels failed to show any effect (278).

These conflicting results may originate from differences between studies in blood sampling conditions causing stress in some studies but not in others, and thus affecting the plasma Prol levels found (279). However, no effect of HF on Prol was observed when blood was serially sampled from permanent cannulas throughout the estrus cycle without the animals being stressed due to handling or etherization, but possibly with stress caused by individual housing (280). Some effects during certain phases of the estrus cycle have been reported. At present it is not clear that Prol itself plays a significant role in the effect of fat on mammary carcinogenesis.

7.1.2 Studies in man.

Studies in man have also contributed to our understanding of the interaction between diet and hormones. Fasting or malnourishment may decrease the conversion of androgens into their metabolites and lower their secretion in women

(281,282). Vegetarian women have lower urinary levels of estriol and total estrogens, lower plasma Prol levels and higher plasma SHBG levels, when compared with matched non vegetarian women (102). It is well established that Prol in man is secreted in high concentrations during the early morning hours, which appears to be related to deep sleep patterns during that period (283). The nocturnal levels of plasma Prol were higher among women who consuming a typical Western (omnivorous) diet than among vegetarians (284). Additional studies in the USA and other countries have confirmed that Prol secretion is significantly increased by consumption of Western diets (77). Further support is found in a study that compared the hormone profiles of Bantu women with those of white South African women (285). Prol levels were significantly higher among the white women on a western diet (40% of total energy from fat) than among the Bantu on their customary diet (± 15% of energy from fat) (160). On the other hand, in a study to assess the effects of diet on estrogen and Prol levels in teenage girls in the USA, Chile, Japan and Papua New Guinea, no significant differences in plasma levels of Prol, E_2 or E_1 were found, suggesting that dietary intake of meat and fat does not significantly affect these hormones (286). Some differences in urinary profiles were seen, but these could not be correlated to any dietary variable. In a study with vegetarian and non-vegetarian pre-menopausal Adventist women it appeared that Prol levels in non-vegetarians but not in vegetarians were positively correlated with dietary energy, protein, total and saturated fatty acids and oleic acid (287). Conflicting results have been obtained in studies with BC patients and women at high risk of developing BC (95). Antiprolactin drugs seem only occasionally successful in inducing remission (78). At present there is no direct evidence for a link between diet-dependent high plasma Prol levels and human mammary carcinogenesis. Nevertheless, the results of some of the studies mentioned above and of other reports (77,288) are suggestive of a causal relationship.

7.2 Diet and estrogens.

Slight but significant increases in total serum estrogens have been reported in rats on a 20% lard HF diet but only during metestrus-diestrus (58). In a study comparing the influence of low (0.5% w/w), moderate (5.0% w/w) and high (20% w/w) levels of fat (corn oil) on E_1 and E_2 in rats it appeared that levels of these estrogens were significantly reduced in the LF diet group, but

only during proestrus (158). HF diets influenced neither serum E_2 nor Prog in female SD rats (207). Indirect evidence of a possible link between HF diet and ovarian function is provided by early vaginal opening and estrus in rats on HF (289,290).

7.3 Diet and other hormones.

Besides Prol and estrogens, several other hormones relevant to BC have been reported to be influenced by diet. Increased levels of thyroxine after feeding HF have also been reported (291), but a clear relationship between thyroid, diet and mammary carcinogenesis has as yet not been established. It is not likely that HF diets influence serum GH (291) or corticosterone (291) levels in serum, or pituitary growth hormone synthesis and secretion (273). Increased ACTH secretion resulted in decreased growth of murine mammary tumors (292, 293), but this seemed not to be influenced by HF intake (291). With HF diets the secretion of insulin in rats may decrease (291,294) as well as the binding of insulin to adipocytes (295), while in other studies unaltered (296,297) or elevated (297) serum insulin levels were found. These divergent results make a role of insulin in mammary carcinogenesis uncertain.

The metabolism of nutrients, the synthesis and activity of several hormones, and the metabolic activity of adipose tissue suggests common areas of interaction that may favor carcinogenesis. For example many nutrients participate in the citric acid cycle, and acetate, as a precursor of cholesterol, is the primary precursor of all steroids. Thus interactions between diet, steroid hormones (estrogens, androgens) and Prol are conceivable. More than one nutrient or enzyme may affect the activity of regulating hormones of pituitary or hypothalamic glands that controls someone's hormonal status. At the same time adrenal hormones may be involved as well (298).

8. POSSIBLE MECHANISMS OF DIETARY FACTORS.

There appears to be no doubt that mammary carcinogenesis in rats and mice is inhibited by caloric restriction and enhanced by a HF diet (299). What appears to remain unknown is the mechanism of action of the HF diet and caloric restriction. It most likely operates through a variety of pathways. Food restriction significantly reduced average tumor number and size by the end of

a 26-week study but treatment for 8 days with E_2-benzoate (1 μg/rat) significantly increases mammary tumor incidence despite underfeeding (300). Postulated mechanisms fall into two categories, namely those involving direct effects of fat on tumor development and those involving indirect effects on metabolism. Furthermore, nutritional elements may differ in their effects during initiation and promotion.

8.1 Direct effects.

Modification of membrane structure, permeability and function. High levels of dietary fat markedly influence the lipid composition of the tumor, qualitatively and quantitatively reflecting the fatty acids of the consumed diet. Cell membrane composition may influence a number of cellular physiologic events, such as membrane fluidity, receptor availability, protein mobility, prostaglandin biosynthesis and degradation, and other chains of biochemical events. A positive relationship between dietary LA levels and prostaglandin biosynthesis has been reported (301). Additionally it has been reported that indomethacin is able to block the stimulatory effect of HF (302,303). This observation that the tumor growth-promoting effect of a HF diet *in vivo* is undone by indomethacin treatment, suggests that products of the prostaglandin synthetase pathway may stimulate tumor growth either directly or indirectly. It is known that DMBA-induced rat mammary tumors synthetize much more PGE_2 than does normal mammary tissue (304). The mechanisms that play a role in the differences in effect of unsaturated oils from saturated fats may depend on differences in biosynthetic production and availability of prostaglandins (179).

Modification of intercellular components, including enzymatic regulatory systems and transport mechanisms. An other mechanism of dietary fat may be the intake of PUFAs that are subject to *in vivo* peroxidation which may result in damage to (macro)molecules. The peroxide content of tissues and peroxidation itself have been postulated to play a role in carcinogenesis (305). Diets high in unsaturated fat increase tissue peroxide contents by increasing the degree of unsaturation of membrane lipids and rendering them susceptible to peroxidation. This peroxidation can be reduced by vitamin E or selenium, indicating interactions between the type of dietary fat and antioxidants in determining the risk of cancer.

Enhanced mitotic activity. Fairly convincing evidence for a direct effect of dietary fat on mammary tumor cells has been derived from studies with cell cultures. Upon the addition of unsaturated fatty acids enhanced mitotic activity was observed, expressed as reduced cell doubling time (215). This was also the case for normal rat mammary gland cell cultures. Maximal growth stimulation of either normal or neoplastic cells required both hormones (insulin, Prol and hydrocortisone) and unsaturated fatty acids. This together with the fact that Prol appeared to influence the fatty acid composition of the mammary gland fat pad (215), may lead to assume that Prol has a dual role in mammary carcinogenesis: directly on the mammary epithelium and indirectly through the fat pad.

8.2 Indirect effects.

Modification of the endocrine status. Diet may change the critical hormonal milieu mainly by increased hormone secretion by the pituitary and the ovaries, but also by changing hormone receptor populations, changes in binding capacity and activation of aromatase systems. However, from a study with ovariectomized rats it appeared that both Prol and estrogens are required for hormonal interaction to promote tumor formation. The increased tumor incidence at HF intake may also be explained by continuously increased estrogen levels in the body. Two observations support this hypothesis: first, enhanced conversion of androstenedione into estrone, resulting from increased aromatase and 17β-hydroxysteroid dehydrogenase activity in fatty tissues of mammary pads and peripheral tissues (306); and second, increased intestinal β-glucuronidase activity, resulting in a substantial contribution to the plasma estrogen pool by enterohepatic absorption, due to enhanced intestinal bacteria content and activity induced by a HF diet (27). The possibility of alteration of hormonal status through dietary fat in man has been stressed repeatedly. Hill (307) suggested a mechanism whereby dietary fat may influence estrogen patterns. He showed that subjects on HF diets produce feces with metabolically very active anaerobic bacterial flora. In addition, a HF diet led to increased amounts of biliary steroids in the colon. Anaerobic gut bacteria are able to produce E_1, E_2 and 17-methoxy-E_2 from bacterial metabolites of cholesterol. Gut bacteria *in vitro* have been shown to produce steroid estrogens from bile acids and cholesterol metabolites, and variations in dietary fat have been shown to affect both the composition of the intestinal flora and the levels of fecal

steroid metabolites (308). Sufficient data suggest that nutrition affects pituitary function and, through this or more directly, the production of estrogens and other hormones (309).

Effects on cellular and humoral immune systems. Several studies suggest that dietary fat can markedly influence immune responsiveness. Diets with a high content of PUFA appear to be immunosuppressive, as compared with saturated fat (310). Additionally, hyperlipidemia can affect macrophage activity adversely (310) and reduce peripheral lymphocyte concentrations (311). In a study with SD rats it was found that the modulating effect of fat on the immune system depends on the duration of feeding and the type of fat consumed (312). However, the interactions of dietary fat and immune system are not fully understood yet.

Modification of formation and absorption of carcinogens in the gastrointestinal tract, and influencing molecular events. Alterations in fecal flora and bile acid metabolism resulting in conversion of bile acids into estrogens by particular enzyme systems as indicated earlier.

Effect on cell-to-cell communication. It has been hypothesized that intercellular communication plays an important role in control and differentiation of growth of normal and of neoplastic tissues, and that interruption of intercellular communication is a possible mechanism in tumor promotion (313). This proposal is based on the fact that many tumor promoters block a specific type of cell-to-cell communication, metabolic cooperation, in which small regulatory molecules and/or ions are transferred between adjacent cells via gap junctions (313). Unsaturated fatty acids (LA and oleic acid) block metabolic cooperation in vitro in Chinese hamster V79 cells. The major cell-cell interaction in the mammary gland after birth is between epithelium and adipocytes. Mammary adipocytes release both saturated and unsaturated fatty acids. Studies with both intact animals and mammary organ cultures indicate that Prol stimulates this process by activating lipase in mammary adipocytes (215,314). Once released, unsaturated fatty acids are either reprocessed by fat cells or taken up by the epithelial cells, whereas saturated fatty acids are removed from the gland by circulation. Probably unsaturated fatty acids are needed for mammary cell growth, while saturated fatty acids inhibit the growth of both normal and neoplastic mammary epithelial cells (315).

Food derived carcinogens. In view of the known properties of o-methylarylamines of causing colon cancer and BC in male and female rats, it is possible that such a compound formed during the frying of protein-containing

foods may be the actual carcinogen responsible for these cancers (172). It has also been proposed that the mutagens found in the charred surface of fried beef provide the carcinogens involved. Mutagens have been identified in fried foods (316). However, to date there is insufficient evidence for involvement of specific mutagens.

9. RECENT DEVELOPMENTS IN BREAST CANCER RESEARCH.

9.1 Growth factors and breast cancer.

Growth and differentiation of the mammary gland has long been considered to be largely under endocrine control. The involvement of paracrine and autocrine regulation has received little attention until recently. In general, endocrine substances are produced and secreted in a specific cell type and reach their target cells through circulation, whereas paracrine agents are released near their target cells and thus reach them by local diffusion. It has been proposed that important aspects of growth control of tumors are mediated by cancer-cell-secreted factors which could either be autostimulatory (autocrine factors) or stimulatory for surrounding tissues such as connective tissue or vessels (paracrine factors) (317). These growth factors (GF) can be under endocrine control, but it has not been proven that endocrine stimulation of BC necessarily involves paracrine or autocrine stimulation at all. However, in a strain of mice with a high incidence of mammary tumors it has been demonstrated that continued growth partly depends on EGF (318).

It is believed now that GFs are proteins which by definition, act from outside the cell, and play an essential role in the regulation of normal and neoplastic growth. They are naturally occurring polypeptides, which cause the cell to increase in size and/or in number (319). The GFs best characterized to date include nerve growth factor (NGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), plateled derived growth factor (PDGF), transforming growth factor α and β (TGFα and TGFβ), insulin, and somatomedins (insulin-like growth factors; IGFs). Studies on differential growth responses of several types of mammary epithelium during the normal estrus cycle showed that local GFs, produced by cells of the fat pad, may be responsible for the growth of mammary terminal end-buds (320).

Several laboratories have reported the identification and isolation of mammary cell GFs that may act in a paracrine fashion. However, most of these factors have not been well characterized with regard to their structure and physiological function. Growth induced by EGF is mitotic, it induces cell division. To promote cell division EGF increases the rate of transport of uridine, sugars, putrescine and alanine and it activates several metabolic steps (321). It also triggers signal transduction pathways resulting in an increased intracellular calcium content and an increased pH, acting as a sort of second messenger. GFs are believed to act through high-affinity specific cell membrane receptors (322), inducing or promoting proliferation activity of the responding cell. The receptor is an integral 170-kDa membrane protein with an extracellular binding domain that serves to bind the ligand, a transmembrane region, and an intracellular domain facing the cytoplasm. Binding of GFs to their receptors is affected by prostaglandins (323). GFs do not use cAMP as a second messenger but seem to act synergistically with agents that increase cAMP levels in their target cells (324). Binding of GFs to receptors results in phosphorylation of several proteins (325). It has been suggested that the EGF receptor has both protein kinase and auto-phosphorylating activity (326). The rat mammary tumor-derived mammary tumor factor (MTF) enables normal rat kidney cells to grow in soft agar (327). This MTF may be related to EGF since they compete for binding to the same EGF receptor.

TGF is strongly involved in the autocrine growth of tumor cells. It includes at least two functionally and structurally distinct groups of factors, TGF-α and TGF-β; to date even three diffent TGFβ proteins are known. TGF-α binds to and interacts through the EGF receptor (328). High-affinity cell surface receptors for TGF-β have been found in a variety of cell types. In contrast to TGF-α, which is a potent mitogen, TGF-β can inhibit the growth of all epithelial cells. TGF-β can antagonize the biological effects of TGF-α and many other GFs. Cooperative or antagonistic interactions between TGF-α and TGF-β may be determined by a balance in the synthesis and secretion of these proteins or other GFs (329). TGF-β is one of the most interesting growth regulating peptides because it has been demonstrated to both stimulate and inhibit cell proliferation according largely to cell type (330).

9.1.1 Hormones and growth factors.

A role of estrogens in BC has been clearly established as indicated earlier (see also ref. 331). Estrogens are mitogens for BC cells, but the exact mechanism of their action has not been elucidated by now. Estrogens can directly alter gene expression at the level of messenger RNA concentrations (331). As a result of this estrogenic influence on gene expression estrogen-responsive BC cells secrete specific proteins. Some of these secreted proteins could serve as autocrine and paracrine growth-regulating activities with multiple actions, resulting in tumor promotion. Studies of cloned cell lines of human BC have confirmed that E_2 can serve as a proximate mitogen for mammary cells. Physiological concentrations of E_2 have been shown to promote nucleotide incorporation into DNA and to increase thymidine labelling index, net DNA synthesis and cell number. E_2 induced a large number of enzyme activities specifically involved in nucleic acid synthesis (331). Apart from regulation of essential growth-regulating enzymes, estrogens also alter the activity of several other proteins, including PR (332).

The synthesis of GFs can be stimulated by glucocorticoids and estrogens (333,334). There is some evidence that the level and action of GFs are influenced by the nutritional status. EGF is involved in the induction of normal differentiation of the mammary gland (335). The presence of EGF receptors in human breast tumor tissue is highly indicative of a progressive disease (336). Other important GFs known to be involved in BC are IGF-I (=somatomedin-C) and TGF-α as growth stimulators and TGF-β as a growth inhibitor. E_2 does not stimulate IGF-I secretion though glucocorticoids and anti-estrogens, which both inhibit the growth of MCF-7 cells, strongly inhibit IGF-I secretion (331).

ER- tumors may contain a population of BC cells the growth of which is primarily regulated by GFs such as EGF and not by estrogens. On the other hand, ER+ tumors may contain BC cells producing EGF like peptides, such as TGF-α, the synthesis of which may be estrogen-controlled (337,338). Estrogens regulate the production of TGF-α in human BC cell lines that are ER+ and growth responsive to E_2, while ER-, non-estrogen-responsive cells produce these factors (338). EGF-like activity, probably TGF-α, as well as IGF-I activity and EGF are markedly regulated by E_2 (339). TGF-β is inhibited by estrogens and induced up to 30-fold by antiestrogens (340). EGF may influence 17β-hydroxy-steroid-dehydrogenase activity (341). Because the E_2 concentration

in breast tumor tissue is significantly higher than in normal breast tissue (342), an increased conversion of E_1 to E_2 in breast tissue adjacent to the tumor could be one mechanism by which an increased E_2 concentration is achieved and made available for tumor growth promotion. Together, estrogen/antiestrogen regulation of BC may involve a coordinated regulation of multiple growth-stimulating and growth-inhibiting, autocrine and paracrine GFs. Studies with cell cultures indicate that a substantial fraction of estrogen-induced tumor growth is mediated at the level of alterations in secreted polypeptide GFs. This obviously has potential therapeutic implications.

9.2 Oncogenes and breast cancer.

The mechanism of cancer remains elusive despite intensive research. In the past decade investigation of retrovirusses as well as gene transfer techniques have been used to reveal the presence of transforming genes in animal and human tumors. These studies have resulted in the isolation and characterization of a number of transforming genes, generically designated viral oncogenes (v-oncogenes) and cellular oncogenes (c-oncogenes). The large majority of c-oncogenes has been discovered by the aid of retroviruses (343,344) and hybridization studies have demonstrated that most oncogenes in human tumors are related to oncogenes of retroviruses (345). About 50 distinct oncogenes have been identified to date. Counterparts of oncogenes in normal cells are known as proto-oncogenes which are preserved by nature (344,346). Proto-oncogenes play a key role in the growth control of cells, coding for polypeptide structures including proteins involved in important stages of proliferation and differentiation. C-oncogenes are activated c-proto-oncogenes. Proteins encoded by oncogenes have at least four different functions: (1) GFs, (2) GF receptors, (3) role in signal transduction (G-protein like and protein kinases), and (4) nuclear proteins involved in regulation of gene expression.

The identification of oncogenes, specifically those activated in mammary tumors, may eventually be helpful in examining the mechanisms underlying mammary carcinogenesis and hormonal modification thereof.

One may assume that they all can play a role in the machinery that transduces the GF signal to the cell nucleus includes the GF receptors, their substrates, a number of key enzymes, cytoskeletal protein, transcriptional

factors, DNA-binding proteins, and a complex of enzymes that channel deoxy- and ribonucleotide precursors into the growing forks of DNA replication (347). Some of the more convincing work linking oncogenes and growth factors has also elucidated part of the relationship between GFs and cancer. A proto-oncogene, c-sis, codes for the B-chain of PDGF, and c-erbB codes for the EGF receptor (348). Moreover, there is evidence suggesting that several oncogene products are similar to GF receptors in that both have transmembrane and tyrosine kinase domains. The p21 ras oncogene product probably is involved in transduction of a GF signal and may be an obligatory intermediate in this pathway. GFs have been shown to increase transcription of certain proto-oncogenes (myc and fos), the products of which may in turn regulate the transcription of other genes necessary for stimulation of cell proliferation. All in all, this suggests that many oncogene products may be involved in GF-receptor response pathways and may indicate that alterations in those products and pathways can occur, leading to the development of neoplastic transformation (348). However, the concept of autocrine or paracrine regulation implies that malignant transformation may not be only the result of excessive production and action of positive GFs, but also the failure of cells to synthesize or respond to specific negative GFs they normally release to control their own growth (349), or that may be present in circulation.

9.2.1 Oncogenes in NMU-induced mammary carcinomas.

In many human cancers certain activated oncogenes, identified as members of the ras oncogene family have been found in different amounts. This group of at least three structurally different but related genes, H-ras, K-ras, and N-ras codes for proteins of 189 amino acid residues, generally known as P21 (350). The ras genes acquire their transforming properties by single point mutations in two domains of their coding sequences, most commonly in codons 12 and 61. These membrane-associated proteins bind GDP and GTP and have an intrinsic GTPase activity (346). These proteins exist in equilibrium between an 'active' state in which they have bound GTP and an 'inactive' or 'relaxed' state in which they bind GDP (346). Point mutations altering ras proto-oncogenes to oncogenes block this GTPase activity, thus preventing normal deactivation of these proteins (351).

Activated H-ras-1 genes have also been identified in NMU-induced rat mammary carcinomas (350). Molecular characterization of the genes revealed a single

point mutation: the twelfth codon was GAA in stead of GGA of the normal allele, encoding glutamic acid in stead of glycine (350). Each H-ras-1 oncogene in NMU-induced tumors appeared to be activated by the same G-->A transition (352). Substitution of NMU by DMBA as the carcinogen in the rat mammary tumor system however, led to a dramatic decrease of the percentage of tumors carrying activated H-ras-1 oncogenes, from 86% to 23% (346). DMBA, unlike NMU, forms large adducts with guanine and adenine residues leading to unspecific mutations. Each of the H-ras-1 oncogenes from DMBA-induced mammary tumors exhibited a normal codon 12. Their activating mutations were localized in the two adenine residues of codon 61. This indicates that NMU is directly responsible for the activation of H-ras-1 oncogenes in codon 12, presumably through the generation of miscoding O^6-methylguanine adducts. It also would imply that the process of oncogene activation must take place concomitantly with, and presumably contribute to, initiation of carcinogenesis. Ras activation however, may also occur during tumor progression and be a secondary event that provides full growth potential to become fully malignant (353).

However, it is not very clear what makes an intravenous injection of NMU exclusively induce mammary carcinomas. The active state of proliferation of the developing mammary gland at the time of carcinogenic insult most likely plays a fundamental role leading to fixation of the promutagenic O^6-methylguanine adduct to form a permanent mutation. It is likely that endogenous hormonal status is important in this respect, since ovariectomy of rats before NMU-injection reduces tumor formation. The important role of the various ras genes in carcinogen-induced animal tumors has been summarized recently (353,354).

9.2.2 Oncogenes in human breast tissue.

Many studies on the structure or expression of cellular oncogenes are based on established tumor cell lines. Amplified and over-expressed myc genes have been reported in one fifth of all breast cancer lines screened (355). Activated H-ras has been found in cells from a breast carcinosarcoma but not in normal cells from the same patient (356). Several oncogenes have been found to be expressed in four primary tumors (357). Investigations on primary breast tumors, which have the advantage that oncogene findings can be related to tumor histological and behavioral parameters, are scarce. In 62 human

specimens frequency and level of expression of several oncogenes (c-myc, c-H-ras, c-K-ras and c-N-ras) were greater in breast carcinoma tissue than in benign breast tissue (358). The contribution of altered ras genes to development of human tumors has been summarized (359). Amplification of the HER-2/neu oncogene in primary breast tumors is a predictor of both over all survival and time to relapse in patients with breast cancer (360). Amplification of the neu (c-erbB-2) oncogene in human mammary tumors is often accompanied by amplification of the c-erb-A oncogene (361). However, its meaning is not clear yet.

A link between oncogenes and steroid hormone receptors has been suggested recently. This theory is based upon the assumption that steroid receptors interact with nuclear DNA at enhancer regions that regulate the transcriptional activity of structural genes (351) and has been supported by the recently discovered structural kinship between the v-erb-A gene, possibly related to thyroid hormone, and the genes encoding the receptors for steroid hormones (362,363).

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CHAPTER 3

EFFECTS OF DIETARY FAT AND ETHER ANESTHESIA ON PLASMA LEVELS OF PROLACTIN, ESTRADIOL-17β, CORTICOSTERONE AND PROGESTERONE IN FEMALE F344 RATS

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ABSTRACT——The influence of dietary fat and method of sacrifice on plasma values of prolactin, estradiol-17β, progesterone and corticosterone was investigated under well defined conditions in healthy female F344 rats. Animals were fed equi-energetic semipurified diets differing in both amount (5 versus 20% by weight) and type of fat (sunflower seed oil versus lard). The results indicate no influences of dietary fat on plasma levels of prolactin and estradiol-17β. During proestrus, progesterone levels were higher in rats on high and lower in rats on low sunflower seed oil, both in comparison with rats on high and low lard. Sunflower seed oil was associated with higher levels of corticosterone than lard, irrespective of the amount of fat and stage of the cycle. Large effects of ether anesthesia were found, in comparison to decapitation after adaptation to the handling-procedure. These effects varied during the different phases of the estrus cycle, and were particularly marked during met-diestrus. It is concluded that differences in plasma levels of prolactin and some steroid hormones as reported in several publications are more likely to be due to insufficiently standardized experimental procedures than to amount and type of dietary fat in relation to their suggested role in chemically induced mammary carcinogenesis. Of major importance for a correct interpretation of hormonal data in rodent studies are the use of handling-habituated animals, avoidance of anesthetics, and the accurate confirmation of the stage of the estrus cycle.—

ABBREVIATIONS USED: NMU=N-Methyl-N-Nitrosurea; DMBA=7,12,-dimethyl-benz(a)-anthracene; Prl=prolactin; E_2=estradiol-17β; Prog=progesterone; Cc=corticosterone; ANOVA=analysis of variance; O=estrus; OM=estrus-metestrus; M=metestrus; MD=metestrus-diestrus; D=diestrus; DP=diestrus-proestrus; P=proestrus and PO=proestrus-estrus.

INTRODUCTION

From studies using the NMU- and DMBA-induced mammary cancer model in rats, consuming different amounts of fat, it has been concluded that a high fat intake resulted in enhanced tumor incidences. This phenomenon has been hypothesized to result from increased plasma Prl levels (1). In animals bearing NMU-induced tumors, fed a high fat diet, elevated Prl and total estrogen levels were observed as well as a higher Prl/E_2 ratio during the metestrus-diestrus phase of the estrus cycle (1). In studies using DMBA for tumor induction, high fat levels in the diet resulted in enhanced Prl and E_2 levels in proestrus (2,3). However, another study reported no differences in hormone levels in DMBA treated rats, irrespective of the stage of the estrus cycle (4). Recently, studies with intact, chronically cannulated rats did not show any differences in serum Prl values at any time during the estrus cycle in rats due to differences in the level of dietary fat (5,6). Thus literature is conflicting about the question whether dietary fat can influence hormonal status in female rats.

The discrepancies mentioned may be due to differences in procedures and experimental conditions, particularly differences in blood sampling methods. In several studies blood was sampled after ether anesthesia (1-3), which may lead to acute elevation of plasma Prl levels in both male and female rats (7-10). In other studies, blood was sampled without the use of anesthetics (4-6). Handling procedures were not standardized in these latter studies which may have resulted in differences in stress. Also, stress cannot have been completely avoided in the studies with cannulated rats (5,6) due to the individually housing, repeated blood sampling and perhaps the mere presence of the cannula. Reported effects of dietary fat may have resulted from different types of stress applied to the animals: pharmacological (ether vapor, anesthesia), emotional (cage transport, exposure to novel environment) or physical (animal handling, blood sampling). Furthermore, the cycle dependent and diurnal variation of hormone levels must be taken into account. Misinterpretation of vaginal smears may have been responsible for some of the above indicated discrepancies. Also differences in composition of the diets may have been responsible in part, such as differences in the type of fat used. Moreover, discrepancies may have been caused by strain differences and by the fact that most data were collected from tumor-bearing animals.

The present paper reports a series of studies undertaken to obtain more detailed information on the influences of amount and type of dietary fat and the effect of ether anesthesia during bloodsampling on plasma levels of Prl, E_2, Prog and, as indicator of stress, Cc in female F344 rats, that were not treated with carcinogen.

MATERIALS AND METHODS

Animals and animal care.— In the first study (study A) female F344/CrlBR rats (5 weeks old) were purchased from Charles River U.K. Ltd., Margate, Kent. In the second study (study B) female F344/Cpb rats (21-28 days old) were purchased from TNO-CPB, Zeist, The Netherlands. After computerized randomisation the animals were housed in groups of 4/cage in stainless steel suspended cages with wire mesh bottoms, in a temperature and humidity controlled room (23±1°C, relative humidity at least 40%) with a light-dark cycle of 12 hours. The animals had free access to tap water and were fed the experimental diets ad libitum.

Diets.— The four diets consisted of four possible combinations of quantity (5 and 20% by weight) and type (unsaturated and saturated) of fat. Lard was the source of saturated fat (mainly saturated fatty acids) while sunflower seed oil was the source of unsaturated fat (mainly poly-unsaturated fatty acids). The diets were prepared according to the principles outlined by Newberne et al. (11). Assuming that rats will tend to consume equal amounts of energy when fed diets with different energy density, resulting in differences

in food intake, the diets were composed in such a way that equal intake of the non-variable nutrients in these studies was assured. In Table 1 the ingredient and nutrient composition of the diets is presented, as well as their energy density and the energy contribution of the various nutrients in each diet. The amounts of minerals and vitamins were added at approximately 150% of the nutritional requirements of rats (12). The diets were prepared every 4 weeks and stored at 4°C until fed. The food in the cages was changed twice a week. From every new batch samples were taken for dietary analyses, performed according to standard procedures to check for correct composition. The fatty acid composition of the dietary fats was measured by gas-chromatography (linoleic acid content of sunflower seed oil: 67.0%, lard: 10.0%). The selenium content of the diets (from the protein) was 0.45-0.60 mg/kg. The soyprotein contained less than 5 mg/kg trypsine inhibitor activity. Under the above conditions there were no detectable changes in levels of peroxides and vitamins A and E with time (data not shown).

Experimental Design.— The animals were fed the experimental diets for a period of 8 weeks. Food consumption and body weights were recorded weekly. The animals were habituated to being handled daily and made familiar with the anesthesia chamber (without ether) and the decapitator. During week 8 of the experiments, estrus rythmicity was determined by means of vaginal smears, taken at 8.30 am in order to detect the metestrus and at midday to detect the proestrus stage of the cycle. In study A the animals were sacrificed during all stadia of the estrus cycle by decapitation after standardized exposure to ether. In study B animals were almost exclusively sacrificed in either metestrus (minimal prolactin secretion) or proestrus (maximal prolactin secretion). Two animals per cage were sacrificed by rapid decapitation with a guillotine (Harvard Bioscience, South Natick, MA) in absolute silence, and two were decapitated after standardized exposure to ether (Ether bubbler M22001, Draegerwerk AG, Luebeck, Germany). From animals supposed to be in proestrus another vaginal smear was taken at about 3 pm and the animals that were in proestrus were sacrificed at 4:30 pm. Animals in metestrus were sacrificed at 11:30 am. For confirmation a vaginal smear was also taken from all animals directly after death. The same person performed handling habituation, vaginal smears, anesthesia procedures and decapitation. Based on the cytology of the vaginal smears, stained according to Papanicolaou (13), the cycle was subdivided as follows: O, OM, M, MD, D, DP, P and PO.

Plasma hormone measurements.— Blood was collected from the trunk in heparinized tubes (5000 IE/ml, Kabi, Stockholm, Sweden). Plasma was centrifuged with use of Sure-sep (General Diagnostics, Morris Plains, New Jersey, USA) in a cooled centrifuge at 1250 g during 10 minutes. Plasma was stored at -30°C until assayed. Plasma levels of Prl, E_2, Prog and Cc were estimated by radioimmunoassays.

For rat Prl a homologous double antibody radioimmunoasay was developed using antiserum and standards from the Rat Pituitary Hormone Distribution Program, which were generously provided by Dr. A.Parlow, N.I.A.M.D.D., N.I.H., Bethesda, USA. The procedure used was based on the method described by Kwa et al.(14). Samples were assayed in duplicate at three different dilutions with phosfate buffer to cover the wide range of expected Prl levels. Briefly, 100 μl of diluted plasma, pools and standards were mixed with 50 μl of tracer (NEX-108 r-PRL-[125]I, 170pg; NEN, Du Pont, 's-Hertogenbosch, The Netherlands) and 250 μl antiserum (NIAMDD anti-r-PRL-S-8., final tube dilution 20,000x). Incubation with the first antibody was carried out at 4°C for 6 days. After addition of the second antibody (Donkey-anti-rabbit precipitating serum, RD-17, Wellcome, Beckenham, UK; final dilution 50x) precipitation of the antibody-antigen complex was performed at 4°C for 24 hours. Two ml of ice-cold saline was then added followed by immediate centrifugation for 30 minutes at

TABLE 1.- Composition of the diets

Ingredients	LUF[α]	LSF[α]	HUF[α]	HSF[α]
Casein	13	13	15.4	15.4
Soy protein isolate	13	13	15.4	15.4
Lard	-	5	-	20
Sunflower seed oil	5	-	20	-
Wheat starch	30	30	18.8	18.8
Sucrose	30	30	18.8	18.8
Cellulose	5	5	6.45	6.45
Mineral mix [β]	3.5	3.5	4.5	4.50
Vitamin ADEK mix [γ]	0.3	0.3	0.39	0.39
Vitamin B mix [δ]	0.2	0.2	0.26	0.26
Total	100.0	100.0	100.0	100.0

Composition in weight % and calories per 100 gram consumed food after analysis

Nutrients	LUF[α]		LSF[α]		HUF[α]		HSF[α]	
	%	cal/100g	%	cal/100g	%	cal/100g	%	cal/100g
crude protein[ε]	23.0	92.0	23.3	93.2	27.3	109.2	26.9	107.6
crude fat	6.1	54.9	5.7	51.3	20.6	185.4	20.7	186.3
carbohydrate	61.5	246.0	61.6	246.4	42.9	171.6	43.0	172.0
total ash	3.7	-	3.7	-	4.7	-	4.7	-
moisture	5.7	-	5.7	-	4.5	-	4.6	-
total	100.0	392.9	100.0	390.9	100.0	466.2	100.0	465.9

[α] LUF=low unsaturated fat; LSF=low saturated fat; HUF=high unsaturated fat; HSF=high saturated fat.

[β] Mineral mix contained (in g/kg mix): potassium dihydrogenphosphate: 399; calcium carbonate: 389; sodium chloride: 142; magnesium sulphate: 58; ferric sulphate septahydrate: 5.7; zinc chloride: 0.9; copper sulphate pentahydrate: 0.8; magnese sulphate dihydrate: 4.6; cobalt chloride sextahydrate: 0.02; potassium iodide: 0.007; potassium chromic sulphate dodecahydrate: 0.08.

[γ] Vitamin ADEK mix contained (g per kg mix): mix of vitamin A (2100 IU/g) and vitamin D3 (700 IU/g; Farmills, Putten, The Netherlands), 939; vitamin E, 15 and Menadon sodium bisulphite, 1.0 (Merck, Darmstadt, Germany); wheat starch to make one kg.

[δ] Vitamin B mix contained (g per kg mix): thiamin hydrochloride: 3.0; riboflavin: 2.25; pyridoxinhydrochloride: 4.5; niacin: 15.0; calcium panthothenate: 6.0; biotin: 0.075; folic acid: 0.75; vitamin B12: 0.0375; choline chloride (50.0% to make 1 kg (all:Merck, Darmstadt, Germany).

[ε] (N*6.25).

1500 g. Radioactivity of the sediment was counted in an automatic gamma counter (1270 Rackgamma II, LKB-Wallac, Turku, Finland). The amount of Prl in samples and pools was calculated using Spline Fit Function. Sensitivity of the assay is approximately 50 pg/tube. Interassay coefficient of variation was assessed by pooled sera at different dilutions and was 7-12% (n=15).

Plasma E_2 levels were determined by a modified method based on the procedure described by Eriksen (15). Briefly, samples, pools and standards were subjected to Extrelut-column chromatography. E_2 was extracted with freshly destilled ether and evaporated to dryness. BSA-Tris buffer, containing [2-,4,6,7-^{3}H]-17β-E_2 (NET-317; NEN, Du Pont, 's-Hertogenbosch, The Netherlands) and antiserum (rabbit anti-E_2; TNO-CIVO) were added to duplicate aliquots. After mixing, the tubes were incubated overnight at 4°C. Separation of bound and free E_2 was achieved with dextran-coated charcoal (0.5% Norit A and 0.05% dextran T-70 in BSA-Tris buffer). From the supernatant 0.5 ml was counted. The amount of E_2 in the samples and pools was calculated with the logistic four parameter model. Sensitivity of the assay, using 0,5 ml of plasma, was ca. 2 pg E_2/ml plasma. Recovery of added E_2 proved to be better than 96%. Interassay coefficient of variation was between 15% for 10 pg/ml and 10% for 100 pg/ml (n=10).

For the simultaneous assay of Prog and Cc an extraction and purification procedure was developed using only 100 µl of sample. The method was based on the procedure described by Manlimos et al. (16). Plasma samples, pools and standards were diluted with BSA-Tris buffer and heated during 10 minutes at 70°C to inactivate binding globulines. The samples were then applied to glass-columns, filled with ethyleneglycol coated chromosorb. After equilibration Prog and Cc were eluted with a step-wise gradient of ethylacetate in iso-octane, collected in borosilicate tubes and evaporated to dryness. For Prog the residue was solubilized in BSA-Tris buffer. Out of this extract duplicate aliquots were mixed with buffer containing [1,2,6,7-^{3}H]-Prog (NET-381; NEN, Du Pont, 's Hertogenbosch, The Netherlands) and antiserum (rabbit anti-Prog; TNO-CIVO). For Cc the residue was solubilized in gelatine-Tris buffer. Out of this extract duplicate aliquots were mixed with buffer containing [2,4,6,7-^{3}H]-Cc (NET-399; NEN, Du Pont, Den Bosch, Netherlands) and antiserum (rabbit anti Cc-21-thyroglobulin serum; Miles-Yeda Ltd., Rehovot, Israel). Prog and Cc-assays were incubated either for 90 minutes at 37°C or overnight at 4°C. After incubation the tubes were placed in a water bath at 4°C for 30 minutes. Separation of bound and free steroid was performed with dextran-coated charcoal. The amounts of Prog and Cc were calculated using Spline Fit Function. The recovery of added steroid was 100±2% for Prog and 96±4% for Cc. Sensitivity of the assays were 2 pg/tube, and 10 pg/tube for Prog and Cc. Interassay coefficients of variations were 5-10% for Prog and 10-15% for Cc at concentrations of 2-40 ng/ml for Prog and 20-1000 ng/ml for Cc (n=10).

Statistical analysis.— Growth of the animals, expressed as their body weight-gain (differences in body weights from day 42 to day 77), food intake and food conversion efficiency were analyzed by ANOVA, taking into account amount of fat, type of fat, assay (study A versus study B) and their mutual interactions. For hormonal data three inorthogonal analyses of variance were carried out following logarithmic transformation of the data for each of the four hormones. For study A the ANOVA model consisted of terms for the effects of the amount of fat, type of fat, the stage of the cycle at the time of sacrifice and their mutual two-factor interactions. For study B the model was extended with terms for the method of sacrifice and its interactions with amount and type of fat. Finally, an inorthogonal ANOVA was carried out on the lumped data for the rats in the two studies sacrificed after ether anesthesia, with terms for the effects of assay, stage of the cycle at time of sacrifice, amount of fat, type of fat and their mutual two-factor interactions.

RESULTS

Growth and food consumption.

Data on growth of the animals, their food intake and the food conversion efficiency are presented in Figure 1 and Table 2. The growth of all four groups showed about the same pattern. Results of analysis with ANOVA were as follows. The weight gain was significantly higher for the unsaturated fat groups, as compared to the saturated fat groups (Table 2). Statistically significant differences in food intake and food conversion efficiency were observed if they were expressed in grams food consumed per rat/week and gram weight gain/gram food intake/rat/week, respectively. Animals on low fat (groups LUF and LSF in Table 2) consumed slightly more food and showed a somewhat lower food conversion efficiency than the animals on high fat

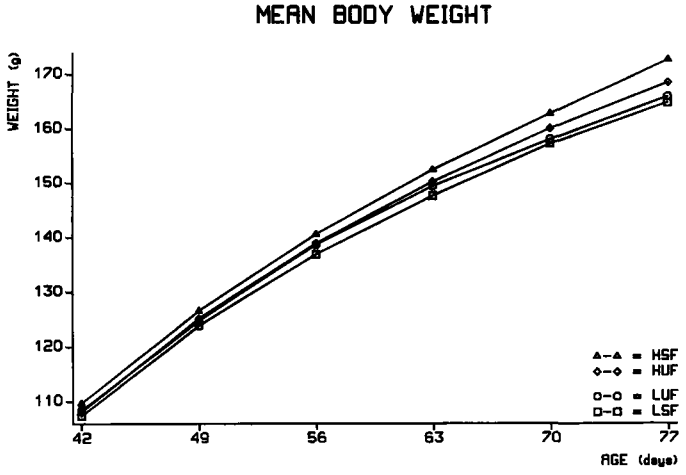

FIGURE 1. Mean body weights (grams) between day 42 and 77 of age.

(groups HUF and HSF in Table 2) diets. Additionally, the animals on saturated fat exhibited a slightly lower food intake than those on unsaturated fat. However, when food intake and food conversion efficiency data were based on calories rather than on grams of food consumed, there were no significant differences (Table 2).

Plasma hormone levels.

Means of plasma hormone values grouped by study, by dietary group and by method of sacrifice are presented in Figure 2. In addition, data for Prl are presented in Table 3. A summary of the statistical analyses is given in Table

4. In this analysis all main variables have been tested, adjusted for all other main variables, and all two factor interactions, adjusted for all main effects but not necessarily for all other two factor interactions.

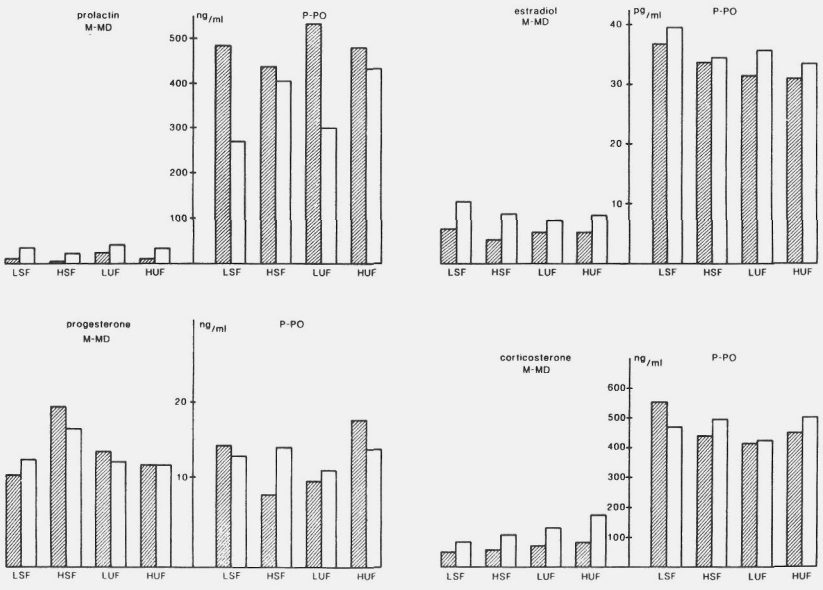

FIGURE 2. Mean hormonal values of Prl, E_2, Prog and Cc $\pm$ standard error of the mean per group during M-MD and during P-PO. The hatched bars indicate levels after rapid decapitation while open bars represent data after ether anesthesia. Data are grouped per dietary group LSF (low saturated fat), HSF (high saturated fat), LUF (low unsaturated fat) and HUF (high unsaturated fat). Levels of significance for differences are indicated in Table 4. The number of observations is as follows:

	after rapid decapitation		with ether anesthesia	
	M-MD	P-PO	M-MD	P-PO
LSF	6	13	13	17
HSF	2	11	13	9
LUF	5	12	15	18
HUF	5	14	14	13

TABLE 2.- Weight gain and overall caloric intake and food conversion efficiency (=FCE) in female F344 rats, fed diets that differ in type and amount of fat, between 42 and 77 days of age.

Study	Diet[1]	n[2]	Weight Gain (grams)	Food Intake grams	Food Intake calories	FCE grams/gram	FCE grams/kcal
study A:	LSF	24	63.7	423.2	1654	0.151	38.5
	HSF	24	64.3	369.4	1721	0.174	37.3
	LUF	24	69.3	447.6	1758	0.154	39.3
	HUF_3	24	72.5	386.2	1801	0.188	40.3
S.E.D.			2.6	8.8	37	0.004	0.9
study B:							
M-MD	LSF	16	71.8	414.4	1620	0.173	44.3
	HSF	16	78.3	371.2	1729	0.211	45.3
	LUF	16	71.8	426.7	1676	0.168	42.9
	HUF_3	16	70.6	356.0	1659	0.199	42.6
S.E.D.			2.4	8.6	38	0.004	1.0
P-PO	LSF	32	72.6	420.4	1643	0.173	44.2
	HSF	32	80.4	366.1	1706	0.220	47.2
	LUF	32	75.7	418.8	1645	0.181	46.0
	HUF_3	32	80.6	359.3	1675	0.224	48.1
S.E.D.			1.9	6.1	27	0.003	0.7

[1] LUF=low unsaturated fat; LSF=low saturated fat; HUF=high unsaturated fat; [2]HSF=high saturated fat.
[3]n=number of animals per group.
S.E.D.=the standard error of difference between means.

Prl.—Analysis of neither study A nor study B (M-MD and P-PO) revealed significant effects of either amount or type of fat on plasma Prl values after correcting for the stage of the cycle. Rats fed the various diets showed rather similar plasma Prl levels throughout the estrus cycle with peak Prl values at proestrus. Prl levels during M-MD tended to be higher in the low fat groups than on high fat. No such tendency was seen in study A or in P-PO. Prl levels at stages other than proestrus ranged from 0.5 to 60 ng/ml. The analysis of the data of the animals in M-MD revealed a marked effect on Prl of the method of sacrifice. The mean plasma Prl values after ether anesthesia (41.3 ng/ml) were significantly higher than after decapitation (8.1 ng/ml). On the other hand, the analysis of the data of the animals in P-PO revealed no effect of the method of sacrifice. No other statistically significant effects were observed (Table 4). The observed Prl levels and their cyclic variation were in line with the literature (7,17).

TABLE 3.- Means of PROLACTIN values[α,β]

Stage of estrus cycle[γ]	Method of Sacrifice[ε]	LSF[δ] Diet		LUF[δ] Diet		HSF[δ] Diet		HUF[δ] Diet	
		Study A	Study B	Study A	Study B	Study A	Study B	Study A	Study B
O	E	27 (3)		60 (2)		57 (4)		20 (3)	
	D								
OM	E	17 (10)		16 (9)		13 (9)		24 (9)	
	D								
M	E	13 (6)	38 (4)	9 (4)	43 (6)	13 (4)	23 (7)	10 (5)	48 (6)
	D		13 (4)		7 (3)		4 (5)		6 (5)
MD	E		37 (3)		43 (2)		5 (1)		71 (1)
	D		7 (3)		17 (2)				
D	E								
	D								
DP	E							7 (1)	
	D								
P	E		157 (12)	180 (2)	161 (8)		299 (7)	100 (1)	306 (8)
	D		425 (10)		396 (8)		185 (8)		320 (11)
PO	E	174 (3)	460 (3)	178 (5)	506 (4)	164 (3)	798 (1)		416 (3)
	D		504 (5)		387 (6)		446 (5)		527 (4)

[α]Prl values are expressed in ng/ml; calculated as antilogarithms of the means on natural logaritmic scale.
[β]The number of rats is indicated in parenthesis.
[γ]The estrus cycle is subdivided as follows: O=estrus, OM=estrus-metestrus, M=metestrus, MD=metestrus-diestrus, D=diestrus, DP=diestrus-proestrus, P=proestrus and PO=proestrus-estrus.
[δ]LUF=low unsaturated fat; LSF=low saturated fat; HUF=high unsaturated fat; HSF=high saturated fat.
[ε]Blood sampling after ether anesthesia is indicated by the capital E, while sampling after rapid decapitation is indicated by a D.

E_2.——No effects of either amount or type of fat, or of the method of sacrifice on E_2 levels were observed, irrespective of the stage of the cycle. The expected cyclic levels of E_2 were found with higher concentrations at proestrus compared to the other stages of the cycle (7,17). However, during P-PO a statistically significant difference was observed between P (31.4 pg/ml) and PO (36.4 pg/ml) animals. In addition, an interaction between amount of fat and cycle was observed (Table 4).

Prog.——High fat tended to be associated with high Prog levels in study A when compared with low fat. In study B (M-MD) no effects of either amount or type of fat or method of sacrifice on plasma Prog values were observed. In P-PO there was no effect of the method of sacrifice. However, an interaction of amount and type of fat significantly influenced Prog values (Table 4) in P-PO. Unsaturated fat at a low level in the diet was associated with decreased

plasma Prog levels, while unsaturated fat fed at a high level seemed to increase plasma Prog levels, both when compared with saturated fat at both a high and a low level. Cyclic changes in Prog levels were in concordance with literature (7,17).

Cc.—Cc was significantly influenced by the experimental conditions in study A. Both type of fat and phase of the cycle (Table 4) significantly influenced plasma levels of Cc. Unsaturated fat was associated with higher levels of Cc in comparison with saturated fat (Table 4). In study B, however, no such effects on Cc were observed. In P-PO, but not in study A and in M-MD,

TABLE 4.- Summary of P values[α] of the 3 inorthogonal analyses of variance for Prl, E_2, Prog and Cc

Hormone	Study	Main variables[β] A	T	C	S	Two factor interactions[γ] AT	AC	AS	TC	TS	CS	Pooled sd[δ]
Prl	A:	-	-	***	n.a	-	-	n.a	-	n.a	n.a	0.75
	B:											
	M-MD	0	-	-	***	-	-	-	-	-	-	0.88
	P-PO	-	-	***	-	-	-	-	-	-	-	0.87
E_2	A:	-	-	**	n.a	-	*	n.a	-	n.a	n.a	0.70
	B:											
	M-MD	-	-	-	-	-	-	-	-	-	-	0.41
	P-PO	-	-	**	-	-	-	-	-	-	-	0.28
Prog	A:	0	-	-	n.a	-	-	n.a	-	n.a	n.a	0.97
	B:											
	M-MD	-	-	-	-	-	-	-	-	-	-	0.66
	P-PO	0	-	***	-	**	-	-	-	-	-	0.84
Cc	A:	0	**	*	n.a	-	-	n.a	-	n.a	n.a	1.06
	B:											
	M-MD	-	-	-	-	-	-	-	-	-	-	1.09
	P-PO	-	-	-	*	-	-	-	-	-	-	0.56

[α]legend : - = P> 0.10
0 = 0.05 < P< 0.10
* = 0.01 < P< 0.05
** = 0.001< P< 0.01
*** = P< 0.001
n.a = not applicable

[β]Main variables defined as: A=Amount of fat (5 versus 20% by weight; T=Type of fat (saturated versus unsaturated); C=stage of the cycle (P-PO vs M-MD); S=method of sacrifice (ether anesthesia versus decapitation without decapitation).

[γ]Two factor interactions as defined in footnote β.

[δ]pooled standard deviation on a natural logaritmic scale.

a significant influence of the method of sacrifice on plasma Cc values was observed: samples taken from decapitated animals showed lower values than those after ether anesthesia.

Overall analysis.—After the individual analyses in the separate studies, all data from the studies with rats sacrificed after ether anesthesia were lumped together and analyzed using ANOVA. No effect of either amount or type of dietary fat on plasma Prl was detected. The amount of fat in the diet influenced plasma E_2 levels ($P<0.05$). High fat was associated with lower levels of this estrogen than a low fat diet. Low fat, on the other hand, was associated with lower Prog values than high fat ($P<0.05$). For Cc, a significant difference was observed for both amount ($P<0.05$) and type of fat ($P<0.05$). High fat and unsaturated fat were associated with higher Cc levels than low fat and saturated fat, respectively.

DISCUSSION

The enhancing influence of high fat content diets on rodent carcinogen-induced mammary tumorigenesis has been well established (18). It has been suggested that these stimulatory effects of high fat are mediated through the hypothalamo-hypophysial system by elevated Prl levels (1,2,3). Estrogens have also been implicated (19,20). The present study demonstrates that neither amount (5 versus 20%) nor type of fat (unsaturated, sunflower oil versus saturated, lard) significantly influence serum Prl in rats at any time during the estrus cycle.

Our data, obtained from healthy, non tumor-bearing rats, are in line with results of Aylsworth et al. (5) and Wetsel et al. (6) who collected blood from intact cannulated rats and reported a failure of dietary fat to influence serum Prl levels in rats. In the latter study data were obtained from both healthy and DMBA treated animals, neither of them showing dietary influences upon plasma Prl. Results by Clinton et al. (21) and by Bosland and Wilbrink (22) obtained from healthy animals and by Hopkins et al. (4) from DMBA treated tumor bearing rats, all sacrificed by decapitation without anesthesia, did also not show any influence of dietary fat on plasma Prl. These data and the present results are not in agreement with results of Chan et al. (1,23) and with Ip (2), who found elevated Prl levels in animals on high fat during proestrus. Their data were obtained from healthy rats (23) and from carcinogen

treated, tumor-bearing rats (1,2), while blood was collected under ether anesthesia by heart puncture.

In the present study, the effects on plasma Prl of decapitation with and without ether anesthesia were directly compared. If one considers the Prl values in the present study at P-PO, as depicted in Figure 2, grouped per dietary variable, there is a systematic difference in Prl levels between the high fat and the low fat groups which is, however, not statistically significant. Irrespective of the type of fat, rats on high fat have higher Prl levels than rats on low fat, but only if sacrificed after ether anesthesia and not upon decapitation without anesthesia. This strongly suggests that the feeding of a high fat diet to female rats results in an increased Prl release upon ether-induced stress, as compared with ether-exposed rats on a low fat diet. This ether-induced Prl release is a transient phenomenon (24). Therefore, the difference between rats on high fat and low fat diets can well be significant, as found by Chan et al. (1,23) and Ip et al. (2), or non-significant, as found in the present study, depending on the exact timing of the blood sampling in relation to the start of the ether exposure. Also the method of sampling may play a role in this respect, i.e. heart puncture versus decapitation; the latter method removes instantaneously the source of Prl, the pituitary. Dietary fat does clearly not affect Prl levels if blood is sampled with a method that avoids acute stress, as is evident from the present study and several others (4,5,20-22). The fat effects on ether induced Prl release in female rats during PO, as well as the absence of an effect of fat on Prl in unstressed rats are apparently independent of (ι) the rat strain used, (ιι) the type of dietary fat, (ιιι) treatment with carcinogens, and (ιν) the presence of mammary tumors, because both F344 and Sprague Dawley rats were used in the various studies, as well as a variety of saturated and unsaturated fats, rats treated with carcinogens and intact rats, and rats with and without mammary tumors (the present study, 1-5,20-23).

Stress seems to have paradoxical effect on plasma Prl levels in the female rat. In the morning it induces an increase in Prl and in the afternoon, only under the influence of estrogen, a suppression of Prl and regardless of the method of stress (25). In our study ether inhalation also induced an increase in plasma Prl levels during the morning of metestrus-diestrus and not in the afternoon of proestrus. The latter is in agreement with other findings indicating that the high serum LH and Prl levels on the afternoon of proestrus are not affected by ether vapor stress (10,26). The stress-induced response of

Prl in both handled and non-handled animals was similar but the magnitude differed in one study (27), but in another study handling of female rats lowered serum Prl levels but raised Prl levels in male rats (28). 'Handled' rats could have adapted somewhat and thus be less responsive to portions of the multiple stress used. Rapid decapitation after adaptation to handling and holding the rat in the decapitator is considered by us as relatively free of stress (22).

Differences in reported effects of fat on plasma hormone levels may not only be explained by procedural differences in the collection of blood including the use of anesthetics, but also by differences in the types and levels of fat in the diets. We directly compared the effects of sunflower seed oil and lard as representatives of poly-unsaturated and saturated fats, respectively. No differences in plasma Prl were found when these fats were fed at 5 or 20% w/w. A 0.5% low fat diet, as used in some of the aforementioned studies (1,3), could be marginally deficient in essential fatty acids. Essential fatty acids fed at a deficient level may well result in disturbed Prl metabolism and in cycle irregularities and other hormonal disturbances (29,30). The slight differences in food intake figures found in the present study can be explained by differences in the energy density of the diets: animals on low fat require a higher food intake to meet the energy requirements compared to high fat. These differences did probably not affect our results because equal amounts of energy were consumed by the rats on low and high fat. Our studies were performed in healthy, cycling female rats which excludes effects of the presence of mammary tumors on weight or food intake.

In the present study also E_2 and Prog were studied as steroid hormones implicated in breast carcinogenesis and Cc as indicator of stress. No statistically significant effects of either amount or type of fat upon E_2 during the estrus cycle were observed in our study. This is in line with results of Hopkins et al. (4) and Wetsel et al. (6) but contradicts Ip and Ip (3) who found elevated E_2 levels during P-PO. Bosland and Wilbrink (22) found rats on low fat diets to have higher E_2 levels than rats on high fat diets during metestrus. This is remarkable because they used the exact same diets as this study, in contrast to Hopkins et al. (4), who used coconut oil, sunflower seed oil and menhaden oil, and Ip and Ip (3) and Wetsel et al. (6), who used corn oil in the diets. Its biological meaning is unclear. Besides diet related effects our data do not indicate that there is a relationship between ether-induced stress and E_2. There are no other data on stress and E_2 available.

To our knowledge this is the first study reporting an effect of dietary fat on Prog in female rats. We found a significant interaction of amount and type of fat on Prog levels during P-PO. In the one other study reporting on dietary influences on plasma Prog in cannulated rats during the estrus cycle no effects of the level of fat were reported (6). Only corn oil was studied in that experiment. More studies on dietary fat effects on Prog in rats are needed to validate our findings. However, handling-induced stress or anesthesia are likely to alter Prog concentrations by secretion from the adrenal glands, because Dexamethason depressed these effects on Prog (31).

We could not find any data on possible interactions of dietary fat and plasma Cc in literature. This study revealed that unsaturated fat was associated with higher levels of Cc than saturated fat. This finding needs confirmation. Additionally, we found elevated Cc levels in ether exposed animals in comparison with non-exposed rats. In literature, some attention has been given to the influence of physiological variations on stress-evoked release of Cc from the adrenal cortex (27,32,33). Elevated Cc levels in response to ether have been reported in both male and female rats (33). Reduced Cc levels in handling-habituated rats indicate that experimental procedures can be stressful and that handling-habituated animals can be resistent to handling-induced stress (27). Stress-induced Cc release was more pronounced in Long Evans than in Wistar-ANV rats (32).

Ether anesthesia of short duration did not alter serum levels of testosterone and FSH, but it increased serum LH and Prl levels significantly, rather strain dependent (32,34). Some strains might have a higher treshold of sensitivity for the neuro-endocrine system to stimulation and are more easily habituated to experimental conditions. Variation in basal hormone levels due to sex, circadian rhythmicity or estrus cycle did not alter the pattern of the response of individual hormones to stress, but did markedly influence its magnitude (24), indicating less response in case of physiologically high levels than in case of physiologically low levels. Differences between rats of the Sprague Dawley and Wistar strain in neuroendocrine responses in cycling females in relation to Prl are believed to be of genetic origin and not related to differences in housing or stress (35). Handling and ether anesthesia also induced elevated serum Prl in female hamsters (36). In addition, female hamsters bled under ether anesthesia by cardiac puncture demonstrated higher serum Prl as compared to females decapitated in the procedure room, but only in diestrus and not in the other stages of the cycle. Male hamsters did not

show such effects. In conclusion differences in stress-induced changes in serum Prl levels can be explained by differences between sexes, strains, experimental design such as sampling interval, sampling method, treatment with chemicals or dietary factors.

Sofar it can be concluded that ether exposure is able to induce shifts in hormone secretion, particularly of Prl, in an exposure-time related manner. The central nervous system plays most likely a role in this respect (10, 37,38), including endogenous opioid-mediated neuromodulation (39-42). Dopamine is the predominant inhibitor of pituitary release of Prl (43). Opioid peptides can reverse the inhibitory effects of dopamine directly at the level of the pituitary gland (44), and Prl, in turn, can stimulate dopamine activity (45). One could hypothesize that dietary factors are able to influence the Prl regulating system via neurotransmitters. Some evidence exists on amino acids in relation to catecholamines and Prl, but not on dietary fat (46-49). In addition, also other hormones may be involved in the regulation of Prl release (10,50).

Because the endocrine system is labile to stressful events, such as handling prior to decapitation or blood sampling under ether anesthesia, the potential influence of stress must be given adequate consideration in endocrine related experiments. Thus, studies of hormone levels in relation to dietary modification of mammary carcinogenesis require not only well defined iso-energetic diets, but also standardized procedures to collect blood needed for hormone analyses and standardized procedures for manipulation of the animal prior to obtaining its blood. Rats are macrosmates and perceive mainly olfactory stimuli thus all sorts of olfactory irritation, including smelling of blood, should be avoided. A method of collecting blood in a way devoid of stress is probably decapitation in a separate room after habituation of the animals to handling, transportation and decapitator.

The biological meaning of some influences of dietary fat on plasma levels of Prog and Cc observed in the present study, is not clear. Both method of sacrifice (only in metestrus and not in proestrus) and, naturally, stage of the cycle appeared to be important variables in relation to plasma Prl values. Type and level of dietary fat, on the other hand, did not affect plasma Prl. However, a non-significant higher plasma Prl was found in ether exposed rats fed a high fat diet than in animals on low fat, in comparison with rats that were not anesthetized with ether prior to decapitation. Thus, our data further support the concept that the stimulating effects of high fat diets on mammary

carcinogenesis are most likely not mediated by altering Prl or estrogen secretion, but they also suggest that rats on a high fat diet and rats on a low fat diet can differ in their susceptibility to (ether)stress-induced Prl release, independent of the type of dietary fat.

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CHAPTER 4

THE INTERACTION BETWEEN CONTENTS OF LINOLEIC ACID AND FAT IN THE DIET ON NMU-INDUCED MAMMARY CANCER IN F344 RATS

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ABSTRACT —The effects of diets varying in contents of fat and linoleic acid (LA) on N-nitroso-N-methylurea [(NMU)CAS:684-93-5)]-induced mammary carcinogenesis were assessed in female F344 rats in a 3x3 factorial study design. Nine groups of 30 rats each were fed semi-purified diets containing 10%, 17.5% or 25% fat (by weight). Because of its high linoleic acid (LA) content (77.2% wt/wt), safflower oil was chosen in combination with beef tallow (3.4% wt/wt LA) to achieve 0.85%, 2.13% and 5.31% LA by weight. A 10th group was fed 17.5% fat-13.45% LA. The rats were fed the experimental diets from weaning on and remained on their diets until termination of the study at 32 weeks after a NMU injection was given at 50 days of age. Mammary adenocarcinomas were histologically verified.

Increasing the content of either fat or LA did not markedly influence tumor incidence (% tumor-bearing rats). Increasing the LA content resulted in an increased tumor multiplicity (number of carcinomas per carcinoma-bearing animal). Increasing the content of both fat and LA in the diet resulted in a non-significantly shortened time to first tumor appearance and a higher tumor yield. The group fed the diet with the lowest fat content and the lowest LA content showed the lowest incidence (36.7%), and lowest tumor multiplicity (1.09). The highest tumor multiplicity (2.57) was found in the 25% fat-5.31% LA group. The highest tumor incidence (63.4%) was found in the 17.5% fat-0.85% LA group. Rats that ultimately developed tumors were growing faster in the last week before NMU injection than rats that did not develop tumors. Significantly more rats in diestrus at the time of NMU injection developed tumors, than did animals in all other stages of the estrus cycle. It is concluded that a high LA content of the diet is apparently associated with a higher mammary carcinoma multiplicity. From this study there appears to exist no threshold of LA with respect to its enhancing effect on mammary carcinogenesis for dietary levels between 0.85% and 5.31% wt/wt (1.70% - 12.53% of energy).—

INTRODUCTION

According to epidemiological data, cancer mortality in man shows strong positive correlations with total dietary fat and with animal fat intake, but not with vegetable fat (1,2). The level of dietary fat was shown to enhance the development of both spontaneously occurring (3) and chemically induced mammary tumors in mice (4,5) and rats (6-11). Studies in mice have provided evidence that fat rather than energy, is responsible for enhanced carcinogenesis (12). Increasing the fat content of the diet was almost always followed by an increased incidence and multiplicity of tumors as well as a shorter time to first tumor appearance (9). Dietary fat is also found to affect the growth of transplantable mammary tumors in mice (13,14), but results are limited and controversial (15). Studies with 7,12-dimethylbenz(a)anthracene (=DMBA) -induced rat mammary tumors showed the potential importance of the type of fat: the number of tumors per group and per tumor-bearing animal was greater in rats fed (poly)unsaturated fats (16-20).

It was suggested that saturated fat could be as effective as polyunsaturated fat if only a minimum quantity of polyunsaturated fat was added to the saturated fat (7,21). A certain minimum amount of LA, between 0.5% and 2.1% (wt/wt), in the diet appears to be required to express the promoting effect of fat, i.e. to permit growth of the tumor. This level is higher than the recommended minimum for laboratory rats: 0.3% for females and 0.6% for males (22). One may conclude that the type of polyunsaturated fat, the amount of essential fatty acids (most likely LA), and the level of total fat are important modulators in experimental mammary cancer. Whether and how these factors are interrelated is not clear.

One of the reasons to study the influence of fat and of the LA content more systematically was the fact that most experimental studies reported differ widely in design, in duration and in food composition (23-26). The use of different carcinogens such as DMBA (16,25,26) or NMU (24,27,28) and different and multiple dosage schedules (25,29), may explain the differences. In addition, treatment with the carcinogen at different ages of the animals, e.g. 35 days of age (30) or 50 days of age (24-26) may account for diverging results. Furthermore, rat strain and source related effects may be resposible as well.

The present study presents a systematic approach to establish the possible relationship between percentage of LA and total amount of fat as dietary factors in breast tumor promotion taking body weight gain into account. The NMU-induced breast cancer model in rats (31) was chosen because NMU has direct acting properties and is water soluble. Moreover, the induced tumors exhibit histologic and endocrine features (32) that closely resemble human breast cancer (33,34).

MATERIALS AND METHODS

Animals and animal care. Female F344 rats, 3 weeks of age, were purchased from Charles River Ltd, Margate, Kent, U.K. After computerized randomization to equalize initial weights, 300 animals were assigned to ten dietary groups of 30 animals each (see Table 1 for experimental design). The animals were housed 5 in a cage, in stainless steel suspended cages with wire mesh bottom in a climate-controlled room (21±1°C; r.h. 50%) with a 12-hour light-dark cycle. Individual body weights were recorded weekly and cages were cleaned twice a week.

Composition of the diets. To attain the required LA and fat contents in the diets mixtures of beef tallow and safflower oil were used as the sources of fat. The diets were prepared according to principles outlined by Newberne et al (35). Assuming that rats tend to consume equal amounts of energy when fed diets with differing energy density, resulting in quantitative differences in food intake, the diets were composed such that equal intake of non-variable

nutrients was assured. Table 2 presents the composition of the diets and Table 3 the nutrients. All diets were adjusted to the same level of vitamin E as present in the highest amount of safflower oil used. All dietary ingredients were obtained from commercial sources and the diets were prepared in house. Every 4 weeks 5-kg lots were prepared and stored at +4°C in the dark until used.

Quality control of experimental diets. Every batch prepared was routinely checked for fatty acid composition, fat content, levels of peroxidation and vitamin E and A content. Fatty acid composition of the individual batches of safflower oil and beef tallow was assessed prior to incorporation in the diets. The fatty acid composition was determined by gas liquid chromatography of a methyl ester preparation according to standard procedures (36; Table 4). Peroxidation tests did not indicate deterioration of the diets. It should be noted that rancidity occurs when the peroxide value exceeds 20 meq/kg. Under the conditions mentioned, there were no changes in levels of vitamins A and E with time (data not presented).

Diet administration and food consumption. All animals had free access to tap water and were fed the experimental diets in powdered form ad libitum, through the whole experimental period. Food was offered in feeders of a design that prevents scattering. The food in the cages was changed completely twice a week. Food consumption was recorded to calculate energy intake and changes in food consumption patterns.

Induction of mammary tumors. When 50 days of age all animals received a single intravenous injection in the tail vein with NMU (50 mg/kg body weight, individually adjusted) under light ether anesthesia. NMU wetted with 3% acetic acid (Sigma, St. Louis, MO, USA), stored at -20°C until used was dissolved and diluted with distilled water to give a solution of 10 mg/ml (pH approximately 5), and used within two hours of formulation. A vaginal smear was taken before NMU injection to be able to relate the final tumor response to the stage of the estrus cycle at the time of NMU treatment. Vaginal smears were stained according to Papanicolaou (37). No acute mortality was observed after injection of NMU. During carcinogen exposure the NMU-treated animals were kept in a carcinogen containment area maintained at reduced pressure and provided with a HEPA-filtered air dust exhaust and an air lock entrance. They were housed in disposable plastic cages with sterile sawdust bedding and filtered tops. Personnel wore fully protective clothing, double latex gloves and respirators and showered out, according to the safety regulations of the institute. All waste was incinerated at 850-1200°C. Animals were transferred to their conventional room after a safety period of 4 weeks. From 5 weeks after NMU injection until the end of the study (32 weeks) all animals were examined for palpable mammary tumors.

Autopsy. Animals were autopsied either at the end of the study or in case of a moribund condition. They were rapidly decapitated and carefully inspected for grossly visible tumors. All palpable and non-palpable mammary tumors were dissected and weighed. A representative part of each tumor was saved for histopathology. All other lesions suspected of being a tumor were dissected as well and collected for pathological examination. These tissues were fixed in a 4% aqueous, neutral phosphate buffered formaldehyde and processed to and embedded in a parrafin wax. Sections 5-m thick were stained with hematoxilin and eosin. Histologic diagnosis of mammary tumors was based on the criteria outlined by van Zwieten (38).

Table 1

Experimental design of a factorial study with amount of fat and amount of LA on NMU induced mammary carcinogenesis[a]

		FAT ⟶			
		23.6	37.9	50	ENERGY %
		10	17.5	25	WEIGHT %
LINOLEIC ACID	0.85	LF-LL[b]	MF-LL	HF-LL	
⟶	2.13	LF-ML	MF-ML	HF-ML	
⟶	5.31	LF-HL	MF-HL	HF-HL	
⟶	13.45		MF-VL		
	(weight %)				

[a] 30 animals per group.

[b] Explanation of abbreviations and contribution (wt/wt) of safflower oil and beef tallow to the diet. In the last column the energy contribution (%) derived from LA is presented.

Group	% safflower oil	% beef tallow	% energy LA
LF-LL (low fat - low LA)	0.70	9.30	2.00
LF-ML (low fat - mid LA)	2.40	7.60	5.03
LF-HL (low fat - high LA)	6.80	3.20	12.53
MF-LL (mid fat - low LA)	0.30	17.20	1.84
MF-ML (mid fat - mid LA)	2.10	15.40	4.61
MF-HL (mid fat - high LA)	6.40	11.10	11.50
MF-VL (mid fat - very high LA)	17.50	-	29.13
HF-LL (high fat - low LA)	-	25.00	1.70
HF-ML (high fat - mid LA)	1.70	23.30	4.26
HF-HL (high fat - high LA)	6.10	18.90	10.62

Statistical analysis. Body weight, growth of the animals expressed as body weight gain, food intake, and calculated food conversion efficiency were analyzed by analysis of variance (ANOVA), taking into account amount of fat, amount of LA and their interaction. In addition, body weight gain was analyzed by regression analysis taking into account amount of fat, amount of LA and their interaction as well as final tumor-bearing. Tumor incidence data (end-point analysis), tumor multiplicity (number of carcinomas per carcinoma-bearing animal) and time to first tumor appearance were analysed by Generalized Linear Models (GLM), containing parameters for effects of amount of fat, amount of LA, their interaction and the stage of the estrus cycle at induction. The link functions going with these GLMs were logit, log and identity respectively, and the error distributions were binomial, poisson and normal respectively. Distribution of tumors over the different mammary pads was analysed by χ^2 analysis.

Table 2
COMPOSITION OF THE DIETS.

Ingredients	10 % fat	17.5 % fat	25 % fat	Source
Casein	13.85	15.08	16.32	a)
Soy-protein isolate	13.85	15.08	16.32	b)
Wheat starch	26.36	20.95	15.54	c)
Sucrose	26.36	20.95	15.54	d)
Cellulose	5.33	5.80	6.28	e)
Mineral mixture	3.73	4.06	4.39	f)
Vitamin ADEK mixture	0.32	0.35	0.38	g)
Vitamin B mixture	0.21	0.23	0.25	h)
Fat/oil	10.00	17.50	25.00	i)
	100.01	100.00	100.02	

Sources:
a) De Meierij, Veghel, Netherlands
b) Purina isolate 500 E, Ieper, Belgium
c) Latensteijn, Rotterdam, Netherlands
d) Latensteijn, Rotterdam, Netherlands
e) Solka Floc, Chemimpo, Amsterdam, Netherlands
f) Mineral mixture: 1,5 times the requirement for male rats:

mineral mixture	composition in mg/g
potassium dihydrogen phosphate	399
calcium carbonate	389
sodium chloride	142
magnesium sulphate	58
ferric sulphate heptahydrate	5.7
zinc chloride	0.9
copper sulphate pentahydrate	0.8
manganese sulphate dihydrate	4.6
cobalt chloride sexahydrate	0.02
potassium iodide	0.007
potassium chromic sulphate dodecahydrate	0.08

g) Vitamin ADEK mixture (mg/g):

Vitamin A (2250 IU/g) + Vitamin D3 (750 IU/g)	939	a)
Vitamin E (50 %)	30	b)
Menadion-Na-bisulfite (K3)	1	b)
Wheat starch	30	c)

a) Farmills, Putten, Netherlands
b) Merck, Darmstadt, Germany
c) Wheat starch is added to make 1 gram

h) Vitamin B mixture (composition in mg/g): (All components: Merck,) (Darmstadt, Germany)

Thiamin hydrochloride	3.00
Riboflavin	2.25
Pyridoxin hydrochloride	4.50
Niacin	15.00
Calcium panthotenate	6.00
Biotin	0.075
Folic acid	0.75
Vitamin B12 (0.1 %)	37.50
Choline chloride (50 %)	931.00, to make 1 gram.

i) - Beef tallow: Smilfood, Leeuwarden, Netherlands
   - Safflower oil: Paveocor, Rotterdam, Netherlands

Table 3
CALCULATED NUTRIENT COMPOSITION OF THE DIETS

	10 % fat diet			17.5 % fat diet			25 % fat diet		
Nutrients	weight %	cal.[a]	cal. %	weight %	cal.[a]	cal. %	weight %	cal.[a]	cal. %
Protein	24.09	96.4	25.2	26.24	105.0	25.2	28.40	114.0	25.2
Fat	10.00	90.0	23.6	17.50	157.5	37.9	25.00	225.0	50.0
Carbohydrates	48.82	195.3	51.2	38.32	153.3	36.9	27.80	111.2	24.7
Dietary fiber	5.33		(1.4)[b]	5.80		(1.4)[b]	6.28		(1.4)[b]
Min. and vit.	4.26		(1.1)[b]	4.64		(1.1)[b]	5.02		(1.1)[b]
Moisture	7.50			7.50			7.50		
Total	100.0	381.7	100.0	100.0	415.8	100.0	100.0	449.8	99.9

[a] Cal per 100 g food, calculating 4.0 kcal/g for protein and carbohydrate and 9.0 kcal/g for fat.
[b] g/100 Cal, to indicate equal amounts of minerals and vitamins for all groups.

Table 4
FATTY ACID COMPOSITION OF DIETARY FATS (weight %)[a]

Fatty acid	Carbon chain length: no. of double bounds	safflower oil	beef tallow
lauric acid	12 : 0	0.1	–[b]
myristic acid	14 : 0	0.2	2.8
myristoleic acid	14 : 1	–	0.6
pentadecanoic acid	15 : 0	–	0.7
palmitic acid	16 : 0	7.0	27.6
palmitoleic acid	16 : 1	0.1	2.3
heptadecanoic acid	17 : 0	–	1.4
heptadecenoic acid	17 : 1	–	0.4
stearic acid	18 : 0	2.5	19.8
oleic acid	18 : 1	12.4	39.6
linoleic acid	18 : 2	77.2	3.4
linolenic acid	18 : 3	0.1	0.4
arachidic acid	20 : 0	0.3	–
eicosenoic acid	20 : 1	0.1	0.2
docosadienoic acid	22 : 2	–	0.8
	Total Saturated	10.1	52.3
	Total Mono-unsaturated	12.6	43.1
	Total Poly-unsaturated	77.3	4.6

[a] These are actual analytical values determined by gas-liquid chromatography of a methyl-ester preparation.
[b] – = not detectable.

RESULTS.

Weight gain, food intake and food conversion efficiency. Fig. 1 shows that rats fed the diets with different fat contents demonstrated comparable growth patterns. Food intake and body weight gain decreased immediately after NMU administration (Fig. 1). Animals of the high-fat (HF; 25% by weight) groups were slightly, but significantly heavier throughout the study as compared to the low-fat (LF; 10%) and mid-fat (MF; 17.5%) groups (Fig. 2). Food intake data over the first 77 days of the study showed that animals on LF consumed more food than those on MF and HF. The HF group consumed more energy than either LF or MF groups. Calculated food intake for animals on LF was 8.5 g per day per animal, which equals an average daily intake of about 32.5 kcal, while food intake was 7.9 g (equalling 32.8 and 35.5 kcal) for MF and HF respectively. Food conversion efficiency (=FCE) patterns during the first 11 weeks of the study were similar for all three fat groups. Animals that ultimately developed tumors showed a significantly greater body weight gain in the last week before NMU application than animals that did not develop tumors ($P<0.01$). In the week after NMU application this was not the case anymore.

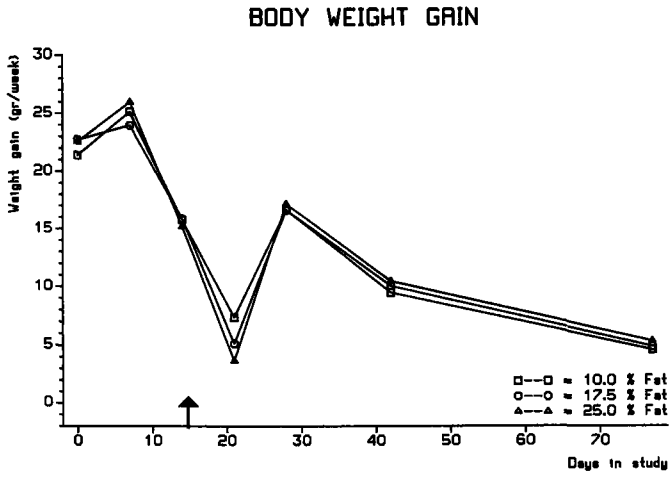

Fig. 1. Body weight gain of the animals during the first 77 days of the study. Arrow: NMU injection. No of animals per point is 30. Data points indicate mean BWG, and SEM did not exceed 3.2% at any point.

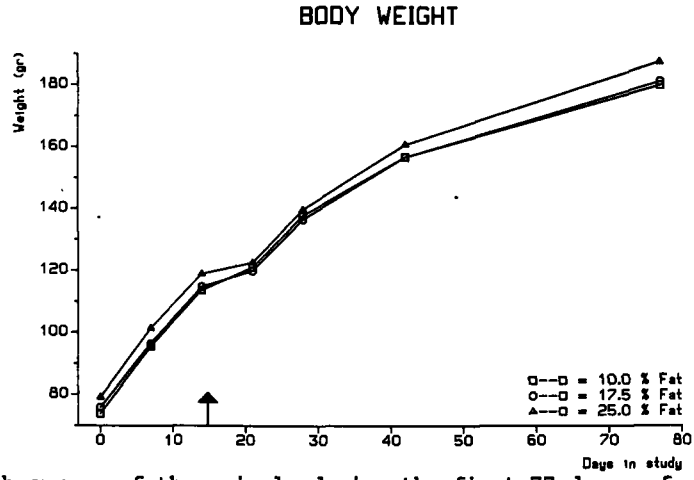

Fig. 2. Growth curves of the animals during the first 77 days of the study. Arrow: NMU injection. No of animals per point is 30. Data points indicate mean BW, and SEM did not exceed 1.4% at any point.

Tumor sites. Mammary tumors were not randomly distributed over the different mammary gland regions. Tumors originated in the right cervical and thoracic region (40%), the left cervical and thoracic region (30%), left abdominal and inguinal (19%) and right abdominal and inguinal region (11%).

Pathology. Data on tumor subclassifications are presented in Table 5. With the exception of mammary tumors and aberrations of organs of the female urogenital tract in general no gross changes in the major organs or organ systems were seen. Most mammary tumors were classified as adenocarcinomas of the tubulo-papillary type (86.1%). During the course of the study 13 animals were sacrificed, mainly due to a moribund condition: one in group LF-LL (nephroblastoma), one in group LF-ML (mammary tumor), one in group LF-HL (pancreatic carcinoma), one in group MF-ML (found dead without tumors), one in group HF-LL (mammary tumor), five in group MF-HL (mammary tumors and/or preputial gland tumors), and three in group HF-HL (one found dead, one with a mammary tumor and one with a sebacceous squamous carcinoma of Zymbal's gland).

Tumor incidence, tumor multiplicity and time to first tumor appearance. The effects of the various diets on mammary tumor development 32 weeks after NMU administration are summarized in Table 6. Data concerning only histologically verified adenocarcinomas revealed tumor incidence to be lowest in the LF-LL group (36.7%). Fig. 3 shows the increase in time of the total number of palpable tumors at low LA diets. The highest final tumor incidence (63.4%), but

not the highest number of adenocarcinomas, was seen in the MF-LL group. The lowest number of adenocarcinomas (12) was found in the LF-LL group, while the highest number (36) occurred in the HF-HL group. Increasing the amount of fat at a constant LA level only slightly influenced tumor incidence; it increased the total number of carcinomas per group significantly ($P<0.01$), but not the number of carcinomas per carcinoma-bearing animal. Fig. 4 indicates that increasing the LA content at a constant level of fat resulted in a significantly increased number of adenocarcinomas per adenocarcinoma-bearing animal ($P<0.01$). Increasing both fat and LA content suggested a shortened mean time to first tumor appearance. However this was not significantly different.

Table 5

Mammary tumors induced by NMU in female F344 rats[a]

Total number of animals: 300 Number of animals with mammary tumors: 146		
type of tumor	Number of tumors	%
ADENOCARCINOMA		
tubulo-papillary adenocarcinoma (AT)	217	86.1
cribriform-comedo carcinoma (AC)	23	9.1
tubulo-papillary adenocarcinoma, regressive (ATR)	4	1.6
compact-tubular adenocarcinoma (SL)	1	0.4
FIBROADENOMA (FA)	7	2.8
	252	100.0

[a] observed over all dietary groups.

Other tumors observed:

keratoacanthomas	8
preputial gland abnormalities (cysts)	3
Zymbal's gland sebacceous-squamous carcinoma	1
soft tissue sarcoma	3
kidney tumor (nephroblastoma)	1
pancreatic carcinoma	1

Table 6
Summary of experimental groups and their final tumor data

Group	Incidence[a]	Adenocarcinomas[b]	Multiplicity[c]	Latency[d]
LF-LL	36.7	12	1.09 ± 0.09	160 ± 13.3
LF-ML	53.4	23	1.44 ± 0.18	153 ± 10.5
LF-HL	40.0	18	1.50 ± 0.19	146 ± 9.7
MF-LL	63.4	33	1.74 ± 0.35	157 ± 9.4
MF-ML	50.0	21	1.40 ± 0.16	124 ± 7.6
MF-HL	53.4	32	2.00 ± 0.40	130 ± 8.1
MF-VL	46.7	25	1.78 ± 0.32	136 ± 10.8
HF-LL	46.7	28	2.00 ± 0.38	136 ± 12.0
HF-ML	40.0	16	1.34 ± 0.26	173 ± 14.9
HF-HL	46.7	36	2.57 ± 0.48	125 ± 7.0

[a]Incidence = % tumor bearing animals, per group.
[b]Adenocarcinomas = total number of histologically verified adenocarcinomas.
[c]Multiplicity = total number of adenocarcinomas per carcinoma-bearing animal ± SEM.
[d]Mean time to first tumor appearance, in days ± SEM.

Estrus cycle. More rats in diestrus-proestrus (vaginal smear analysis) at the time of exposure to NMU were prone to develop tumors than animals in any other stage of the estrus cycle at that time (P=0.02; Table 7). In addition, animals in estrus during exposure to NMU developed only carcinomas of the adeno-tubular type.

Table 7
Stage of the estrus cycle at induction in relation to NMU-induced mammary cancer

Stage of estrus cycle	Number of animals at risk	Final incidence (%) (adenocarcinomas)
Estrus	71	42.2
Estrus-Metestrus	28	50
Metestrus	25	36
Metestrus-Diestrus	37	40.5
Diestrus	36	66.7
Diestrus-Proestrus	33	45.5
Proestrus	40	52.5
Proestrus-Estrus	28	57.1

This table is not corrected for dietary factors.

DISCUSSION

Weight gain and total energy intake showed similar and comparable patterns in all dietary groups. However, our results indicate a clear toxic effect of NMU on food intake behavior. This is expressed by a lowered food consumption, a lower body weight gain and a pronounced decrease in FCE directly after application of the carcinogen. The decrease in FCE is most clear in the HF group.

The results of this study indicate that the variations among the diets used in our study do not have much influence on the incidence of NMU-induced mammary carcinogenesis. There is a slight tendency of increased tumor incidence with increased fat content at constant LA level, but not as pronounced and statistically significant as claimed by others (24,25). Data on mean time to first tumor appearance and tumor multiplicity per group in our study differ slightly from results of others (24). The total number of palpable tumors (Fig. 3) suggests a very clear dose-response relationship with increasing fat content at a constant low level of LA. This seems most clear in the first part of the study (up to about 24 weeks) but less clear in the last part.

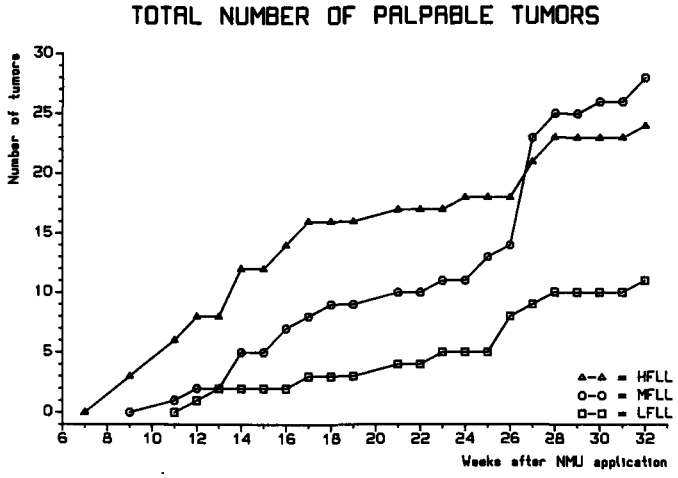

Fig. 3. Effects of LF, MF and HF diets at a constant low level of LA in the actual food on the number of palpable tumors throughout the course of the experiment. Diets were fed from weaning until 32 weeks after NMU administration.

Comparable data on the other levels pointed to the same direction but were not as clear. However, our study of 32 weeks, together with other studies of different duration (23-26), clearly indicates that differences in duration may result in different end-point results leading to different conclusions.

Feeding HF diets to rats generally produces an enhanced tumor response of carcinogen-induced tumors (6-11,39). HF intake has been shown to increase the rate of DMBA- and NMU-induced breast carcinogenesis. On the other hand the enhancing effect of fat may be minimized by restricting calories (40). However, it is still difficult to prove that HF per se is unambiguously primarily responsible for this result. Increasing the fat content directly increases the energy density of a ration (Table 3). In our study animals fed LF consumed the largest mass of food but animals fed HF consumed most energy. It is reported (41-43) that if energy intake is restricted by 20% or more, both tumor incidence and tumor multiplicity will decrease significantly. This is not the case, however, if energy intake is reduced by only 10%. In our study net energy intake was about 8% lower in LF and MF groups than in HF. We therefore suggest that tumor response in our study is determined by fat and/or LA content per se rather than by net energy intake. This is in accordance with another study reporting the highest tumor response in animals on high polyunsaturated fat diets (9). However, our results cannot completely rule out the involvement of net dietary energy intake. It is reported that fat calories are more efficiently utilized for growth relative to carbohydrate or protein calories (44), which is reflected in more energy retention in the carcass of the animal as body fat (45) and explains why body weights of animals fed HF diets were heavier in several studies (44-47). Accidentally, animals of the HF diet group appeared to be slightly, but significantly, heavier from the onset of our study, but it is unlikely that this initial difference accounts for different energy intake during the first 11 weeks of the study. Primarily their body weight gain during this period (121.1±1.29g; mean±SEM) did not differ substantially from the body weight gain of MF (118.1±1.42g) or LF (118.1±1.41g). Secondly, the higher body weights may reflect the enhanced fat utilization as is in line with other studies (44-47).

Evidence for dietary involvement in mammary carcinogenesis based on tumor count data is not very reliable. Tumor count data are subject to uncontrollable variables, primarily because the number of tumors developing per animal varies widely. Multiple tumors in an animal are not independent events. The total number of tumors per group reflects both incidence and multiplicity

and therefore is not suitable for optimal statistical analysis as discussed by Peto et al. (48) and Gart et al. (49). In our study incidence and multiplicity were suitable, separate variables to test, because we had not to deal with differences in survival time or influences of incidental or fatal tumors.

The question whether the type of fat or oil in the diet might be important to breast tumor development has been studied previously. As saturated fats coconut oil (7,50), tallow (21) and lard (21) have been used, which were compared to unsaturated fats such as corn oil (51), sunflower oil (7) and sometimes refined linoleic acid (7,11,50). Some studies did not show any difference in tumor response between rats on mainly saturated and rats on mainly unsaturated fat diets (7). Other studies showed clear effects in mice (5) or in rats (50). The small effect of saturated fat on tumor incidence could be enhanced by addition of LA (7); this effect was not specific for LA because also small quantities of menhaden oil were able to enhance mammary carcinogenesis. Of the fatty acids used, the level of LA in the diet correlated best with spontaneously developed breast cancer (52). Studies in rats with cis and trans isomers of fatty acids excluded the possibility that isomers play an important role (26).

Our data suggest a dose-response relationship between LA content in the diet and tumor multiplicity (Fig. 4). This relationship is more pronounced at a constant low level of fat than at MF or HF and seems to correspond with observations by Cohen et al. (24) for plant oils differing in LA content. We cannot explain why the MF-ML and HF-ML groups had lower tumor multiplicities than we expected. However, we cannot establish a dose-response relationship in a definitive manner, nor do the data support the existence of a threshold level of LA. Chan et al. (27) could not find a significant correlation between tumor yield and LA consumption in a study in the NMU model with HF (25% wt/wt) diets containing different levels of LA from several sources. Ip et al. (25) studied the effect of increasing contents of LA at a HF level (20% wt/wt), achieved by combinations of unsaturated fat of vegetable origin, on the development of DMBA-induced mammary tumors and observed an apparent positive relation between tumor incidence and dietary LA concentration in the range of 0.5% to 4.0% LA (wt/wt). They calculated a breakpoint at 4.2% essential fatty acids (=EFA) for the incidence response and 4.1% EFA for the tumor yield response. Differences in the relative proportions of various classes of fatty acids might provide a partial explanation.

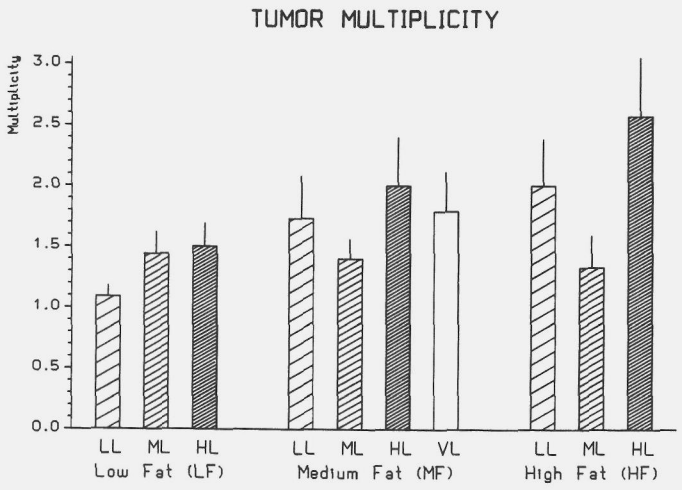

Fig. 4. Effects of the various dietary treatments on tumor multiplicity, expressed as the histologically verified total number of adenocarcinomas per carcinoma bearing animal.

Carroll (23) compared corn oil and menhaden oil at levels of 3, 10 or 20% (wt/wt) in the DMBA model. Increased corn oil intake increased both incidence and number of tumors but higher levels of menhaden oil reduced both. An inhibitory effect of menhaden oil in the NMU model was also reported (23,28). The results of both Hopkins et al. (7) and Ip et al. (25) together, suggest the existence of a saturating level of LA in promoting mammary tumorigenesis. This may partly explain why polyunsaturated fats (PUFAs) are more effective than unsaturated fats in the enhancement of cancer development. Tumor growth rate appeared to be determined partly by the total amount of dietary fat and partly by the level of polyunsaturated fat (53). If once the LA requirement for optimal tumor expression is met, further enhancement of development might depend primarily on the amount and not on the type of fat. Differences between families of fatty acids may influence many of the physiological and pathological aspects of dietary fat (54,55), including desaturation, incorporation into membranes, and enzymatic pathways. Relative proportions of classes of fatty acids in the diet may be of greater importance than the absolute amount of any one class.

Other findings support the hypothesis that the generally reported effects of fat might be related to prostaglandin metabolism or other unclassified biologically active products of PUFAs. LA can serve as a precursor of prostaglandins. So far, the role of prostaglandins is complex and not well understood, but evidence is available that they play a role in tumor proliferation (56,57).

In our study animals were not selected according to the stage of their estrus cycle at the time of NMU administration. Significantly more rats in diestrus at the time of exposure to NMU developed tumors than animals in any other stage of the estrus cycle. This is in line with some data (58), but in contrast to other data suggesting that proestrus or estrus (59) are the prevalent stages of the cycle. The latter observations suggest changes in the hormonal milieu during the estrus cycle to be important for subsequent development of rat mammary tumors.

An as yet unexplained observation in our study was that rats ultimately developing tumors in average grew faster in the week before carcinogen exposure than rats that did not develop tumors. This may be related to the stage of cell division at that time, possibly influenced by endocrine parameters. In addition, in the MF-LL group many animals were seen in diestrus at the time of exposure, and ultimately this group developed the highest incidence of carcinomas. It seems allowable to conclude that the stage of the cycle at the time of tumor induction should be considered carefully.

As can also be concluded from results of our study, there seems to be a topographic site preference in mammary cancer biology. The mammary fat pad has shown to be an immunologically privileged site with respect to transplantable mammary tumors. However, it is unlikely that differences in immunocompetence simply do account for the striking preferential growth of tumors in the right cervical and thoracic region in our study. One explanation could be the natural way of NMU being transported through the body by the blood stream. The tumor locations found then simply reflect the dose of carcinogen carried to the target tissue in charge. But it could also be a reflection of the number and the stage of differentiation of the terminal end-buds, suggested to be the most sensitive target for chemical carcinogenesis (60). It has been observed earlier that there are more terminal end-buds in thoracic than in abdominal mammary glands (60).

It is concluded that a high content of total fat in the diet is associated with a high tumor incidence, while increasing the LA content affects tumor

multiplicity progressively. We could not find a threshold for LA. Our data reflect complex interactions of both total fat and LA content in the diet with the endocrine system in relation to rat mammary cancer.

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CHAPTER 5

INTERACTIVE EFFECTS OF DIETARY FAT AND LINOLEIC ACID ON PLASMA PROLACTIN, ESTRADIOL-17β, PROGESTERONE AND CORTICOSTERONE, AND STEROID HORMONE RECEPTORS IN NMU-INDUCED MAMMARY CANCER IN F344 RATS

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ABSTRACT—The interactive effects of dietary fat and linoleic acid (LA) on endocrine parameters in N-nitroso-N-methylurea-induced mammary cancer were studied in female F344 rats in a 3x3 factorial study design. Nine groups of 30 rats each were fed semi-purified diets containing 10%, 17.5% or 25% fat (w/w) and 0.85%, 2.12% or 5.31% of LA. A 10th group was fed 17.5% fat-13.45% LA. The rats were fed the diets from weaning onwards until the end of the study at 32 weeks post NMU, which was administered at 50 days of age. At the end of the study plasma levels of prolactin (Prl), estradiol-17β (E_2), progesterone (Prog) and corticosterone (Cc), and levels of estrogen- (ER), progesterone- (PR) and androgen receptors (AR) in the tumors were assessed.

The level of fat, but not the LA content in the diet influenced the level of Prl and E_2. The effect of fat on Prl, however, was not the same for all levels of the LA content in the diet. An effect of LA, but not of dietary fat on plasma levels of Prog was observed. No main effect of either dietary factor on Cc levels was observed, only a marginal interaction between both factors. There were no differences in plasma hormone levels between tumor-bearing and non-tumor-bearing animals. The level of fat in the diet did not influence content and affinity of AR, ER and PR in the tumors. However, the LA content of the diet seemed to influence PR content. The AR content was inversely related to the lifespan of a tumor. The PR content and the affinity constant of ER and PR decreased with increasing weight of a tumor.

It is concluded that although certain influences of dietary fat and LA on endocrine parameters in NMU-induced rat mammary carcinogenesis were observed, it is not likely that fat or LA intake per se will influence the biochemical action of Prl, E_2, Prog or Cc. The level of steroid hormone receptors in chemically induced mammary carcinomas appeared to be primarily dependent on the endocrine status of the animal rather than on dietary fat and LA if fed at an adequate level. However, animals fed low to marginal or perhaps deficient levels of dietary fat may exhibit reduced hormone receptor levels in comparison with animals fed HF diets.—

ABBREVIATIONS USED: DMBA, 7,12-dimethylbenz(a)anthracene; NMU, N-nitroso-N-methylurea; Prl, prolactin; E_2, 17β-estradiol; Prog, progesterone; Cc, corticosterone; ER, estrogen receptor; PR, progesterone receptor; AR, androgen receptor; GH, growth hormone; DES, diethylstilbestrol; LA, linoleic acid; HF, high fat; LF, low fat.

INTRODUCTION

Breast cancer is considered to have a multifactorial origin; dietary fat and hormones seem to be important etiologic factors. A positive correlation has been shown between the incidence of mammary cancer and the intake of dietary fat in both women and laboratory animal models (1,2). In addition, interactions between dietary fat and hormones have been implicated in the process of mammary carcinogenesis.

In comparison with a low-fat diet, a high level of dietary fat enhances the development of both spontaneously occurring tumors in mice (3) and chemically induced mammary tumors in mice (4,5) and in rats (6), leading to increased

tumor incidence and multiplicity as well as a shorter time-to-first-tumor appearance (7,8). The effect of saturated fat on chemically induced mammary cancer can be enhanced by polyunsaturated fat (9). It has been suggested that LA and perhaps oleic acid and linolenic acid are most important in the enhancing effect of dietary fat on chemical carcinogenesis in the rat mammary gland. A certain minimal, threshold amount of poly-unsaturated fat is perhaps essential to enhancement of DMBA-induced rat mammary tumors by a HF diet (9).

Mammary cancer development in experimental animals is affected by a number of hormones, in particular E_2, Prog, Prl (10,11), and GH (12). Most mammary tumors in rats resulting from exposure to chemical carcinogens such as DMBA and NMU exhibit a high degree of hormone dependence, and regress after ovariectomy (13), hypophysectomy (14) or during treatment with antiestrogens (15). While in chemical induction models of mammary cancer carcinogens bring about the initiating event, dietary fat and hormones are considered to act primarily during the promotional stage of carcinogenesis. It has been suggested that the hormonal milieu at the time of carcinogen exposure critically influences mammary tumor development (11). The promotional effect of HF diets on mammary carcinogenesis has been suggested to be related to elevated levels of circulating Prl by some authors (15), but others state that this is open to question (11,16). In studies with rats, treated with DMBA for mammary tumor induction, HF diets either elevated Prl and E_2 levels in proestrus as compared with LF diets (17,18), or had no effect (9) suggesting that both Prl and estrogens play a role in concert with fat to produce tumor promotion. Only one study also estimated ER levels in mammary carcinomas in relation to the level of dietary fat. The ER content was found to be lower in DMBA-induced mammary tumors of animals fed dietary fat at a level of 0.5% (w/w) than in tumors of animals fed 5 or 20% of fat (17).

Thus, both the total fat content, the type of fat, the amount of polyunsaturated fat, the essential fatty acid content (most likely LA) in the diet, and the endocrine system are important modulators of experimental mammary cancer. Whether and how these factors are interrelated is not clear. The present study was intended to investigate systematically the possible interrelationship between dietary LA, total fat content in the diet, and the endocrine system in affecting mammary carcinogenesis. Prl, E_2, Prog and Cc (as an index of stress) levels were studied in relation to NMU-induced carcinogenesis. In addition, ER, PR and AR levels in the tumors were estimated simultaneously in the same sample. The NMU-induced mammary cancer model (19)

was selected because NMU can be administered systemically, avoiding possible differences in intestinal uptake and metabolism between groups on different diets, likely with orally administered DMBA. Furthermore, NMU-induced tumors exhibit histologic and endocrine features which closely resemble human breast cancer (20-22). The results of the interaction between contents of fat and LA in the diet on incidence, multiplicity and latency of the NMU-induced mammary carcinomas and data on body weight and food intake have been reported elsewhere (23).

MATERIALS AND METHODS

Chemicals. [2,4,6,7,16,17-^{3}H]-17β-E_2 (s.a 160 Ci/mmol; NET-517), [17α-methyl-^{3}H]-trienolone (=R1881; s.a. 87 Ci/mmol; NET-590), and radioinert R1881 were obtained from New England Nuclear, Du Pont, 's-Hertogenbosch, Netherlands. [^{3}H]Org2058 (s.a. 45Ci/mmol;TRK.629) and radioinert Org2058 were obtained from Amersham, Utrecht, Netherlands. DES (D4628) and Cc (C2505) were obtained from Sigma, St.Louis, MO. All tracers were stored in absolute ethanol at -20°C, and their purity was checked by chromatography. Antisera used were reported elsewhere (24). NMU was obtained from Sigma, St. Louis, MO, USA and stored at -20°C until used. All other chemicals were of analytical grade.

Experimental design, animals, animal care and experimental diets. Details on experimental design, animals and animal care have been reported in detail previously (23). Groups of 30 female F344 rats, kept under well controlled conditions, were fed semipurified diets, ad libitum, from weaning until termination of the study. Mixtures of safflower oil and beef tallow, as summarized together with the experimental design of the study in Table 1, were used to achieve the desired contents of 0.85%, 2.12%, 5.31% and 13.45% LA and 10%, 17.5% or 25% total fat (w/w) offered in semi-purified diets. Diets were prepared according to the principles outlined by Newberne et al (25). Assuming that rats tend to consume equal amounts of energy when fed diets with differing energy density, resulting in quantitative differences in food intake, the diets were composed in such a way that equal intake of the non-variable nutrients in these studies was assured. All diets were adjusted to the same level of vitamin E as present in the diet with the highest amount of safflower oil. All dietary ingredients were obtained from commercial sources and the diets were prepared in house. Every 4 weeks, 5 kg lots were prepared and stored at +4°C in the dark until used. The quality of every batch prepared was routinely checked for fat content, fatty acid composition, levels of peroxidation and vitamin E and A content according to international standard procedures. Body weight gain and food intake were recorded weekly.

Induction of mammary tumors. When the animals were 50 days old, they received a single intravenous injection in the tail vein with NMU (50 mg/kg body weight) under light ether anesthesia. NMU wetted with 3 % acetic acid was dissolved and diluted with distilled water to give a solution of 10 mg/ml (pH approximately 5) and used within two hours of preparation. During carcinogen exposure the NMU-treated animals were, for safety reasons, kept under conditions as reported previously (23). Animals were transferred to their conventional room after a safety period of 4 weeks. No acute mortality was observed after injection of NMU. From 5 weeks after NMU injection all animals were examined for palpable mammary tumors, weekly until the end of the study.

Table 1.

Experimental design of a factorial study with amount of fat and amount of LA on NMU-induced mammary carcinogenesis[a)]

		FAT ⟶			
		15	27.5	40	ENERGY %
		10	17.5	25	WEIGHT %
LINOLEIC ACID	0.85	LF-LL[b)]	MF-LL	HF-LL	
⟶	2.13	LF-ML	MF-ML	HF-ML	
⟶	5.31	LF-HL	MF-HL	HF-HL	
⟶	13.45		MF-VL		
	(weight %)				

a) 30 animals per group.

b) Explanation of abbreviations and contribution (w/w) of Safflower oil and Beef tallow to the diet:

Group	% safflower oil	% beef tallow
LF-LL (low fat - low LA)	0.70	9.30
LF-ML (low fat - mid LA)	2.40	7.60
LF-HL (low fat - high LA)	6.80	3.20
MF-LL (mid fat - low LA)	0.30	17.20
MF-ML (mid fat - mid LA)	2.10	15.40
MF-HL (mid fat - high LA)	6.40	11.10
MF-VL (mid fat - very high LA)	17.50	-
HF-LL (high fat - low LA)	-	25.00
HF-ML (high fat - mid LA)	1.70	23.30
HF-HL (high fat - high LA)	6.10	18.90

Termination of the study. Thirty two weeks after administration of the carcinogen, animals were sacrificed by decapitation with a guillotine (Harvard Bioscience, South Natick, MA, USA). This was done in a quiet room, without ether anesthesia to avoid stress and excitement of the animals as described elsewhere (24). Blood was collected from the trunk in heparinized tubes (5000 IE/ml, Kabi, Stockholm, Sweden) and plasma was separated by centrifugation with the use of Sure-sep (General Diagnostics, Morris Plains, NJ, USA) in a cooled centrifuge at 1200 g during ten minutes. Plasma was frozen and stored at -20°C until assayed. A vaginal smear was taken within half an hour after decapitation to be able to relate hormonal values to the stage of the estrus cycle. Vaginal smears were stained according to Papanicolaou (26), with slightly modification.

Autopsy. Animals were autopsied either at the end of the study or, in case of a moribund condition, during the study, and inspected for gross tumors. All palpable and non-palpable mammary tumors were excised from the animals after decapitation, measured with calipers and weighed. Tumors weighing over 1 gram were cut into pieces. A representative part of each tumor was saved for histopathology. The other part was immediately frozen in liquid nitrogen and stored at -70°C for biochemical determinations. The tumor tissues collected for pathological examination were embedded in paraffin and 5 μm sections were prepared and stained with hematoxilin and eosin. Histologic diagnosis of mammary tumors was performed according to van Zwieten (27).

Plasma hormone measurements. Plasma levels of Prl, E_2, Prog and Cc were determined by radioimmunoassay procedures as described elsewhere (24). Content and affinity of unoccupied ER, PR, and AR were measured by a dextran coated charcoal assay based upon the method originally described by Korenman et al. (28). Data from a 6-point assay were analyzed using the Scatchard plot method (29), the intercept with the abscissa corresponding to the number of hormone molecules bound (Bmax) and the negative reciprocal of the slope of the line ($-1/\alpha$) to the dissociation constant (Kd), which is an estimate of the affinity binding between receptor and ligand. Data were corrected for non-specific binding (30). Receptor concentration was expressed as fmol steroid bound per mg extracted tissue protein.

Hormone receptor estimations. Buffers and solutions were as follows. Basic buffer, pH=7.4 (BB): 10 mM Tris-HCl, 1.5 mM EDTA, 1.5 mM dithiothreitol, 250 mM sucrose, 1.5 mM $MgCl_2$, 20 mM sodium molybdate, 10% glycerol. Homogenization buffer (HB): BB + 1 TIU/ml aprotinin and 0.1 mM Bacitracin. Solubilization buffer (SB): BB + 0.1% BSA. Charcoal solution for AR: BB + 0.1% Dextran T70 and 1% Norit A. Charcoal solution for ER: BB + 0.05% Dextran T70 and 0.5% Norit A. Charcoal solution for PR: BB + 0.005% Dextran T70 and 0.5% Norit A.

Subcellular fractionation was conducted as follows. All procedures were performed at 0-4°C. Frozen tumor tissue was slightly thawed, minced and homogenized in 4 ml HB for 10 seconds with an Ultraturrax. Then 4 ml HB was added and the homogenization procedure was repeated. A cytosol preparation was obtained by centrifugation of the homogenate for 45 min at 105,000 g (Beckman L8-70). From this supernatant aliquots were taken for receptor estimation. In addition an aliquot was taken for measurement of the protein concentration according to Lowry et al (31), using pooled calibrated mammary tumor cytosol as reference standard.

For determination of ER, 100 μl aliquots of cytosol were incubated for 20-24 h at 0-4°C with 0.2-5 nmol 17β-^{3}H-E_2 in the absence or presence of a 100-fold excess of DES to determine total and non specific binding. Separation of receptor-bound and unbound ligand was achieved by incubation with 200 μl charcoal solution for 20 minutes at 0-4°C and centrifuged at 3000 g. Aliquots of the final supernatant were counted in liquid scintillation cocktail (LSC: efficiency 40-50%) to obtain the ER concentration in the cytosol. All samples were processed in duplicate.

For determination of AR, 100 μl aliquots of cytosol were incubated for 20-24 h at 0-4°C with 0.2-5 nmol ^{3}H-R1881 in the absence or presence of a 100-fold excess of R1881 to determine total and nonspecific binding. Binding of R1881 to the PR was inhibited by excess Org2058 (0.72 nmol) added to all tubes in AR analysis experiments. Further processing was similar to the ER procedure except for the particular charcoal solution for AR.

For determination of PR, 100 μl aliquots of cytosol were incubated for 20-24 h at 0-4°C with 0.2-8 nmol ^{3}H-Org2058 in the absence or presence of a 100-fold excess of Org2058 to determine total and non-specific binding. Binding of Org2058 to the glucocorticoid Rc was inhibited by excess Cc (1.8 nmol), added

to all tubes in the PR analysis experiments. Further processing was similar to the ER procedure except for the particular charcoal solution for PR.

Statistical analysis. The plasma hormone levels of Prl, E_2, Prog and Cc were, after log transformation, analysed by regression analysis[2] taking into account amount of fat, amount of LA and their interaction, as well as the final tumor status, the stage of the estrus cycle at termination of the study and their interaction. The ER, PR and AR content and affinity constants of mammary tumors were analysed by regression analysis taking into account the same factors as for the plasma hormones, except the terms with the tumor status. In this analysis all main effects were tested, adjusted for all other main effects as well as all two-factor interactions adjusted for all main effects (but not necessarily for all other two-factor interactions).

Table 2.

Mean plasma levels (± SEM) of Prl (ng/ml), E_2 (pg/ml), Prog (ng/ml) and Cc (ng/ml). Data are not adjusted for stage of the estrus cycle at termination of the study.

Group	Prolactin		Estradiol-17β		Progesterone		Corticosterone	
	mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
LF-LL	8.6	3.7	12.0	1.9	12.0	1.8	369.8	58.6
LF-ML	10.4	2.5	12.2	2.0	9.8	2.7	264.5	39.9
LF-HL	5.1	0.6	10.8	1.4	14.3	2.3	373.5	79.4
MF-LL	35.7	7.9	16.8	2.4	13.7	2.1	485.4	54.3
MF-ML	29.4	9.4	14.7	2.2	11.9	2.2	407.6	71.8
MF-HL	7.2	1.4	12.8	1.3	10.4	2.3	243.1	47.2
MF-VL	43.4	14.8	10.5	1.7	6.0	0.8	342.8	43.5
HF-LL	22.5	15.3	9.7	1.1	12.7	2.3	398.1	70.6
HF-ML	5.9	0.7	10.8	1.3	8.2	1.1	534.4	76.6
HF-HL	18.1	8.8	12.7	2.0	18.3	3.7	441.4	73.4

RESULTS.

The effects of the various diets on mammary tumor development concerning tumor incidences, tumor multiplicity, and latency have been reported elsewhere. Table 2 presents the raw data of plasma levels of Prl, E_2, Prog and Cc grouped by diet. These data do not suggest any dietary influence on plasma hormone levels at all. It should be noted however, that they may be strongly biased by estrus cycle influences. To avoid this bias regression analysis of the data was selected as statistical method. In Tables 3-6 results of this analysis are presented. It should be noted beforehand, that analyses have been performed on log transformed data because untransformed data were not distributed normally and did therefore not allow standard analyses. Inherent to

the regression analysis model, standard deviations of log transformed data are interdependent and can not easily be compared with each other. Therefore, they are not presented in tables.

In Table 3 mean plasma hormone values of Prl, E_2, Prog and Cc are presented for animals on LF-LL, grouped per stage of the estrus cycle at termination of the study and adjusted for all other effects. These values are expressed as the antilogarithms of the fitted mean values derived from regression analysis. Only the LF-LL group is presented here as an example rather than to present the data of all stages separately. The cyclic pattern for all dietary groups however, is comparable with these data, although the respective numerical values differ by a constant factor as a result of the influences of fat and/or LA. Prl and E_2 levels were higher during proestrus and estrus than during the other stages of the estrus cycle (Table 3), as expected (24).

Table 3
Plasma hormone values of Prl, E_2, Prog and Cc, grouped per stage of the estrus cycle, for animals on LF-LL diets. The values are expressed as the antilogarithms of the fitted means derived from regression analysis.

	Stages of the estrus cycle[1]							
Hormones	E	EM	M	MD	D	DP	P	PE
Prolactin (ng/ml)	6.9	3.3	2.9	3.0	3.0	4.8	7.3	7.2
Estradiol (pg/ml)	10.1	9.1	7.3	5.6	6.9	9.6	9.9	18.2
Progesterone (ng/ml)	6.1	10.2	13.2	12.7	11.5	10.7	5.1	5.2
Corticosterone (ng/ml)	276	281	262	159	237	224	483	217
no. of observations	130	34	30	38	31	20	4	4

[1]E=estrus; EM=estrus-metestrus; M=metestrus; MD=metestrus-diestrus; D=diestrus; DP=diestrus-proestrus; P=Proestrus; PE=proestrus-estrus.

In Table 4 mean Prl, E_2, Prog and Cc levels of animals in estrus are presented, grouped by diet and adjusted for the other stages of the estrus cycle. As for Table 3 similar tables can be constructed for all other stages of the estrus cycle (data not presented). Regression analysis revealed a significant, nonlinear effect of the amount of fat in the diet, and a borderline effect of the LA content on plasma Prl (Tables 4.1 and 5). Additionally, a

significant interaction between fat and LA on plasma levels of Prl was found (Tables 4.1 and 5). This interaction was rather complex because at levels of 0.85% and 2.12% LA in the diet the highest levels of Prl were observed in animals on 17.5% fat, whereas at 5.31% LA the Prl levels increased slightly with increasing fat content of the diet.

Tabel 4.
Mean hormonal levels expressed as the antilogarithms of the fitted means derived from regression analysis, for animals in estrus.

table 4.1: Prolactin (ng/ml)

	FAT (%)			
LA (%)	10	17.5	25	MEAN
0.85	6.9	29.1	10.6	12.8
2.12	9.9	20.5	6.0	10.7
5.31	6.6	9.4	12.4	9.1
13.21	-	21.9	-	
MEAN	7.6	17.8	9.2	

table 4.2: Estradiol (pg/ml)

	FAT (%)			
LA (%)	10	17.5	25	MEAN
0.85	10.1	14.0	9.4	11.0
2.12	10.1	12.7	8.8	10.4
5.31	10.4	14.0	11.1	11.7
13.21	-	9.1	-	
MEAN	10.2	13.6	9.7	

table 4.3: Progesterone (ng/ml)

	FAT (%)			
LA (%)	10	17.5	25	MEAN
0.85	6.1	7.2	5.8	6.3
2.12	4.0	5.7	5.1	4.9
5.31	6.2	4.5	7.2	5.9
13.21	-	3.5	-	
MEAN	5.3	5.7	6.0	

table 4.4: Corticosterone (ng/ml)

	FAT (%)			
LA (%)	10	17.5	25	MEAN
0.85	276	446	262	318
2.12	206	273	412	285
5.31	224	179	314	233
13.21	-	273	-	
MEAN	234	280	324	

A significant effect of dietary fat level was observed for E_2 (Tables 3, 4.2, and 5). The observed overall effect of dietary fat showed an increase of plasma E_2 with increasing fat content of the diet from 10 to 17.5%, but a decrease after a further rise of the fat content to 25% at every LA content in the diet.

Table 5.

Summary of the regression analysis for all main effects and the two factor interactions between fat and LA, and between stage of the estrus cycle at termination of the study and tumor-bearing. Results are expressed as P-values[1].

	%FAT	%LA	TUMOR	CYCLE[2]	%FAT*%LA[3]	CYCLE*TUM[4]
ln Prl	***	(*)	-	***	***	-
ln E_2	*	(*)	-	**	-	-
ln Prog	-	*	-	***	-	-
ln Cc	(*)	-	-	-	*	-

[1] ***: P < 0.001
** : 0.001< P < 0.01
* : 0.01 < P < 0.05
(*): 0.05 < P < 0.10
- : P > 0.10

[2] Representing the stage of the estrus cycle at termination.

[3] Representing the interaction between fat content and LA content of the diets.

[4] Representing the interaction between tumor-bearing and stage of the estrus cycle at termination of the study.

A significantly nonlinear effect of LA, but not of dietary fat on plasma Prog levels was observed: animals fed 2.12% LA in their diets had significantly lower plasma Prog levels than animals fed 0.85% and 5.31% LA (Tables 4.3 and 5). Higher levels of Prog were observed during metestrus and metestrus-diestrus than during the other stages of the estrus cycle independent of either dietary fat or LA (Table 3). For Cc, no effects of any of the factors tested were observed, except a marginal interaction between fat and LA (Tables 4.4 and 5).

Table 5 presents a summary of the effects of fat and LA contents of the diet, tumor status, stage of the estrus cycle at termination of the study and two-factor interactions between fat and LA, as well as between tumor-bearing and stage of the estrus cycle at termination of the study on plasma levels of Prl, E_2, Prog and Cc.

There were no statistically significant differences between plasma hormone levels of tumor-bearing and non-tumor-bearing animals (Table 5) from similar groups.

Table 7 shows the ranges and means of mammary tumor ER, PR and AR receptor contents as well as their corresponding affinity constants. Analysis of fat and LA contents in the diet and their interaction, and of the stage of the

estrus cycle at termination of the study on these parameters are summarized in Table 6. No statistically significant effects of dietary fat on content and affinity of ER, PR and AR could be observed. The LA content of the diet influenced PR content and ER affinity constant significantly. However, if besides LA and fat content of the diet also tumor weight and lifespan (see below) of tumors were included in the regression model, these LA effects disappeared.

If the tumors were grouped by weight, the highest number of binding sites for all three receptors was found in tumors between 1 and 2 g, as compared with either smaller (<1g) or larger (>2g) tumors as is illustrated in Figure 1. The AR content was inversely correlated with the lifespan (=duration of the presence of a tumor before termination of the experiment) of a tumor ($r=-0.453$; $P<0.001$; $n=60$; Figure 2). The affinity constant of ER and PR decreased significantly with increasing size of the tumor, while no effect of tumor size was observed on the affinity constant of AR. There were no correlations between plasma hormone levels and corresponding hormone receptor content in the tumors (data not shown).

Table 6.

summary of the regression analysis for the effects of amount of fat and amount of LA in the diet, the stage of the estrus cycle at termination of the study and the two factor interaction between fat and LA on ER, PR and AR content (B_{max}) and on their respective affinity constants (Kd). Results are expressed as P-values[1].

		%FAT	%LA	CYCLE[2]	%FAT.%LA[3]
ER	Bmax	-	-	-	-
	Kd	-	*	-	-
PR	Bmax	-	**	-	**
	Kd	-	-	-	-
AR	Bmax	-	-	-	-
	Kd	-	-	-	-

[1] **: $0.001 < P < 0.01$
[2] * : $0.01 < P < 0.05$
[3] Representing the stage of the estrus cycle at termination.
[3] Representing the interaction between fat and LA contents of the diets.

Table 7.

steroid hormone receptor range, content and affinity in NMU-induced mammary tumors.

	B max[1]			
	Range[1]	n	mean ± sem	Kd[2]
Estrogen Receptor	12.5 - 123.2	72	52.1 ± 3.9	0.19 ± 0.01
Progesterone Receptor	31 - 2375	69	450 ± 53	1.46 ± 0.07
Androgen Receptor	8.1 - 93.3	70	33.7 ± 2.4	0.18 ± 0.01

[1] expressed as: femtomol / mg protein
[2] expressed as: nMol / l ; means ± sem

DISCUSSION

This investigation studies the role of hormones as mediators for the effects of dietary fat and/or dietary LA on NMU-induced mammary carcinogenesis. The results of tumor development have been discussed elsewhere (23). The results on hormonal involvement as published here, present evidence that the postulated influence of neither dietary fat nor LA on NMU-induced rat mammary carcinogenesis is directly mediated by increased plasma levels of Prl, E_2 and/or Prog, or by direct effects on ER, PR and AR. The promoting influence of HF diets on rodent carcinogen-induced mammary carcinogenesis has been suggested to be mediated through the hypothalamo-hypophysial system by elevated Prl levels (9,15), but estrogens and Prog have been implicated as well (10, 32). Chan et al. (33) first reported that female SD-rats fed a HF diet (20%, w/w) exhibited higher serum Prl levels during proestrus-estrus than rats fed a LF diet (0.5%, w/w). Elevated Prl levels were subsequently observed in rats fed HF diets in several studies (9,15,34). Diets containing either high or low levels of lard resulted in higher plasma Prl levels in HF groups of male rats than in LF groups (35). In NMU-treated rats a HF diet resulted in higher absolute levels of Prl and a higher Prl/estrogen ratio than a LF diet (36). In contrast, others have reported that a HF diet did not elevate Prl biosynthesis and blood levels during the estrus cycle (11,37-40). Studies in carcinogen-treated rats indicated that in ovariectomized animals a HF diet also had a mammary tumorigenesis enhancing effect (41). After initial tumor regression, following ovariectomy, tumors regrew at a faster rate and with a greater incidence in rats on a HF diet than in rats on a LF diet.

Conflicting results on increased Prl levels due to HF diets may originate from blood sampling conditions (24). In some studies animals were anesthetized with ether prior to blood sampling (33,36) while in other studies blood was collected from the trunk after decapitation without anesthesia (11,37). As ether is a strong stimulator of Prl secretion (42), the effect of HF on Prl may have been biased. In rats fed a HF diet elevated Prl values were seen in blood sampled after light ether anesthesia but the role of Prl on mammary carcinogenesis itself was unclear (9). Lesions in the median eminence of rats fed LF increased DMBA-induced mammary tumor response to a lesser extent than did HF, whereas serum Prl was higher than at HF (9). It remains difficult to understand these results fully, because DMBA itself can inhibit pituitary functions, stimulate Prl secretion and deregulate the estrus cycle (43). No effect on Prl was observed in studies with serial bloodsampling via permanent cannulas throughout the estrus cycle, thus without stress due to handling or etherization (16,44). Based on the latter results it is doubtful that the enhancing effects of HF on mammary carcinogenesis are exclusively regulated by Prl. More evidence to support the view that Prl does not play such a role comes from other studies that did not find an effect of dietary corn oil content on plasma Prl, Prog and E_2 in female SD rats decapitated without ether anesthesia (45,46). In a study on the interactive effects of dietary protein and fat also no effect of these variables on serum or pituitary Prl was found (46). We have reported previously that neither content (5 versus 20%) nor type of fat (sunflower oil versus lard) significantly influenced serum Prl in healthy, non tumor-bearing rats at any time during the estrus cycle (24). In this study however, Prl levels increased if the proportion of fat increased from 10 to 17.5% followed by a decrease if fat intake was further increased to 25% (Table 4.1). This effect appeared not significantly related to either tumor incidence, multiplicity or latency. The observed interaction of fat by LA was mainly due to the MF-HF group and was therefore not likely to reflect a general rule. Thus, there seems to be sufficient evidence now to conclude unambiguously that Prl itself is no direct mediator in the effects of dietary fat on mammary carcinogenesis (24,16,46,47).

A slight but significant increase in total serum estrogens in F344 rats treated with NMU and on a HF diet (20% lard) was reported at metestrus-diestrus but not at proestrus-estrus (15). Levels of E_1 and E_2 were significantly lower in SD rats on very low fat (0.5% corn oil) at proestrus than in rats on either LF (5%) or HF (20% corn oil; 10). Other studies

demonstrated no influence of a HF diet on either serum E_2 or Prog levels (11, 44). Although we observed a similar effect of fat on E_2 as on Prl (Table 4.2) in this study, our results on E_2 for animals fed 10 and 25% of fat, are in line with the latter two studies and are similar to observations in non-tumor bearing, intact female F344 rats that we reported elsewhere (24). However, we found increased E_2 levels for animals fed 17.5% of fat, indicating that (at least in this study) intermediate levels of fat behave different from low and high dietary fat content. To our knowledge there is no literature available to compare with this result. No clear effects of either dietary fat or LA on plasma Prog and Cc and effects of both hormones on NMU-induced mammary tumors. No comparable data are available to explain the inconsistent effects of dietary fat on Cc in our study. There were no differences in plasma hormone levels of E_2, Prl, Prog and Cc between tumor-bearing and non-tumor-bearing animals.

Therefore a more likely explanation for the inconsistencies in reported differences in plasma hormone levels between animals on LF and HF diets may be the fact that the experimental design, duration and diet composition varied widely among studies (48-51). Furthermore, use of different carcinogens such as DMBA or NMU at different dosage schedules, and the use of different strains of rats may underlie the reported differences (24,52).

It is generally accepted that so-called cytoplasmic ER and PR are important markers for hormone responsiveness in human breast tumors. At the same time other control mechanisms including cAMP, growth factors and prostaglandins, may operate independently of steroid hormones and their receptors. Besides hormones also dietary factors are involved in growth control of chemically-induced mammary tumors as has been shown by a study where a HF diet enhanced the development of DMBA-induced mammary tumors in ovariectomized rats, indicating that fat may operate partly independently from estrogens (53).

To assess the suggested influence of dietary fat and LA via steroid hormones on NMU-induced mammary carcinogenesis at the tissue level, we measured steroid hormone receptors in the tumors. All tumors assayed contained varying levels of cytoplasmic ER, PR and AR (Figure 1). The ER content of NMU-induced tumors was relatively low (52.1±3.9 fmol/mg cytosol protein). This is in line with results for DMBA-induced tumors (42.8±12.3 fmol/mg cytosol protein; mean±SD; n=42) in a study by Leung and Sasaki (54).

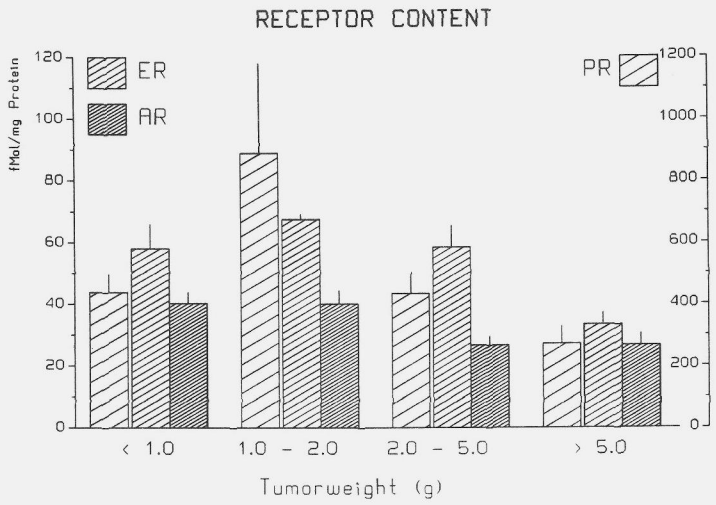

Figure 1. Receptor content of ER, PR and AR, expressed as maximum number of binding sites (fmol/mg cytosol protein) of NMU-induced mammary tumors in F344 rats. Tumors are grouped by weight. Stalks on bars depict SEM.

However, they were somewhat lower than those reported by Williams et al (55) for NMU-induced tumors (37-272 fmol/mg cytosol protein) and for DMBA-induced breast tumors (93-260 fmol/mg cytosol protein) in both F344 and W/ICRF strains. Although part of the variation is undoubtedly due to differences in methodology used in different laboratories, these data indicate a great variability in ER content in chemically-induced rat mammary tumors.

Ip and Ip (10) showed that a 0.5% corn oil containing diet was able to depress serum E_2 and to reduce ER content of DMBA-induced mammary tumors in rats in comparison with animals on diets containing 5% or 20% corn oil. It was concluded that dietary fat did not influence the molecular mechanism of estrogen action. PR levels were not affected by the level of dietary fat consumption in that study, which is consistent with our findings. We observed a marked effect of LA on PR content. However, if the "lifespan" of a tumor and tumor weight were included in the regression analysis this LA effect disappeared, while PR content and tumor weight appeared inversely related (Figure 2). The latter finding may be explained by the observation that HF diets led to earlier tumor development than MF and HF, while high LA in the diet was associated with heavier tumors than medium and low LA diets (23).

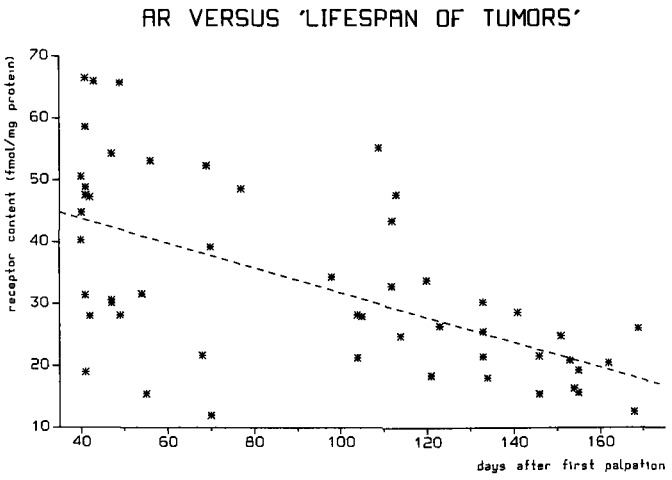

Figure 2. Androgen receptor content decreases with the lifespan (=duration of the presence) of the tumor.

Heavier tumors expressed a lower steroid hormone receptor content than did light tumors, perhaps reflecting a longer lifespan and/or increased hormone independence and an increased tendency to autonomous growth of the heavier tumors. The induction of PR by estrogen has been reported not to be influenced by the level of dietary fat (56). It has also been reported that the level of cytosol ER was low during proestrus and high during all other stages of the estrus cycle, with the nuclear receptor exhibiting a reversed pattern (57). We can not confirm this finding, at least partially due to the limited number of observations in some stages of the estrus cycle in our study. At termination of the study our animals were not selected by the stage of the estrus cycle, as was done by Ip and Ip (10).

The concentrations of AR in our study are in line with another report demonstrating the presence of AR (27.6 fmol/mg protein) in DMBA-induced rat mammary tumors (58). ER, AR and PR may mediate at least part of the possible growth-controlling activity of estrogens, androgens and progestagens in carcinogen-induced tumors, perhaps in concert with each other.

In chemically induced mammary tumors estrogens may be responsible for early events in cellular differentiation and metabolism. Prl is known to be involved in the stimulation of ER synthesis or the activation of existing binding sites in DMBA-induced mammary tumors (54). Therefore, Prl could regulate the

estrogen dependency of mammary tumors. Another study on hypophysectomized tumor bearing rats showed that the growth of NMU-induced rat mammary tumors was dependent on both E_2 and Prl (53). There was a synergistic effect between E_2 and Prl on tumor growth but not on ER, PR or Prl receptor content.

In conclusion, although certain influences of dietary fat and LA on endocrine parameters related to NMU-induced rat mammary carcinogenesis have been observed, it is not likely that fat or LA intake per se result in clear effects on Prl, E_2, Prog or Cc. The levels of steroid hormone receptors in chemically induced mammary carcinomas are perhaps primarily dependent on the endocrine status of the animal rather than on dietary factors such as dietary fat, if fed at an adequate level. However animals fed at low to marginal or perhaps deficient levels of dietary fat may exhibit reduced hormone receptor levels in comparison to animals fed HF diets. Increased Prl levels in (tumor-bearing) rats on HF diets as compared with rats on LF diets reported in the past, are most likely the result of stressful manipulation of the animals during blood sampling rather than a direct result of the HF diet on carcinogenesis vice versa. However, we can not deny an increased Prl level in animals at our intermediate dose of dietary fat. But this was not related to tumor parameters. Although there is little doubt that estrogen and Prl may play a significant role in the genesis, progression and growth of chemically induced rat mammary cancer, our results together with the studies discussed above, strongly suggest that mechanisms other than enhanced hormonal secretion of each of these hormones are involved in the promotion of NMU-induced mammary carcinogenesis in rats by HF diets. Yet, due to the design of our study our data can not exclude important endocrine-related influences on NMU-induced mammary tumor development around the time of carcinogen exposure.

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CHAPTER 6

INTERACTIVE EFFECTS OF TYPE AND CONTENT OF DIETARY PROTEIN ON PLASMA PROLACTIN AND ESTRADIOL-17β, AND ON NMU-INDUCED MAMMARY CARCINOGENESIS IN F344 RATS

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ABSTRACT--The interactive effects of content and type (animal or vegetable origin) of dietary protein on NMU-induced mammary carcinogenesis were studied in female F344 rats in a 3x5 factorial study design. For reasons of breeding, delivery and animal care the study was subdivided into two similar parts, I and II. Two times 15 groups of 16 rats each were fed isoenergetic semi-purified diets containing 15% (LP), 22.5% (MP) or 30% (HP; w/w) of either casein or soybean protein or selected mixtures (80/20; 50/50; 20/80). The rats were fed the diets from weaning onwards until termination of the study at 26 weeks after an NMU injection had been given at 50 days of age. From 5 weeks after injection of NMU onwards, all animals were palpated once weekly, and tumor development was recorded. Mammary tumors were histologically verified. At termination of the study plasma levels of prolactin (Prl) and estradiol-17β (E_2) were estimated. In tumors of selected animals also levels of estrogen (ER) and progesterone receptors (PR) were measured.

Tumor incidence (% tumor-bearing rats) was not influenced by either type or content of dietary protein but it was related to the stage of the estrus cycle at the time of exposure to NMU. Tumor multiplicity (number of tumors per tumor-bearing animal) was dependent on the stage of the estrus cycle at induction ($P<0.001$) and on the type of protein ($P<0.005$). Neither protein content nor interactions between content and type of protein affected tumor multiplicity. Increasing the proportion of vegetable protein at the expense of animal protein was accompanied by a decreased tumor multiplicity. Tumor latency (time to first tumor appearance) was also influenced by the stage of the estrus cycle at induction ($P<0.001$) and by the type of protein ($P<0.01$) comparable with the effect on tumor multiplicity.

The levels of plasma Prl were slightly influenced by the type of protein ($0.1<P<0.05$). In addition, a slight increase of plasma Prl values was observed in animals with multiple tumors. Plasma E_2 levels at the end of the study were not influenced by either content or type of protein individually. However, an interaction of content by type of protein on plasma levels of E_2 was observed ($P=0.01$). Tumors of animals fed casein contained higher ER and PR levels than animals fed soy protein isolate.

It is concluded that dietary protein if fed at levels of 15%, 22.5% or 30% (w/w) at a concomitant relatively high level of fat (17.5%) does not influence the incidence of NMU-induced mammary tumors, although the dose of the carcinogen may mask effects. However, substituting vegetable protein at the expense of animal protein results in a lower tumor multiplicity and a longer tumor latency. The observed effects on plasma levels of Prl and E_2 and the lack of correlation of these effects with either tumor incidence of multiplicity or latency do not indicate that either of these hormones play a clear intermediate role due to dietary protein in NMU-induced mammary carcinogenesis in F344 rats as studied here.

ABBREVIATIONS USED: DMBA, 7,12-dimethylbenz(a)anthracene; NMU, N-nitroso-N-methylurea; Prl, prolactin; E_2, 17β-estradiol; ER, estrogen receptor; PR, progesterone receptor; GH, growth hormone; DES, diethylstilbestrol; LA, linoleic acid; HF, high fat; LF, low fat; LP, low protein; MP, moderate protein; HP, high protein.

INTRODUCTION

Mortality due to cancer of the breast is either remaining steady or continuing to increase in most countries (1-4). Among other factors the risk of developing breast cancer has been related to dietary factors (1-5). The correlation between dietary fat and cancer of the breast is very strong between populations (4,6). However, examination of epidemiological data also revealed positive correlations between breast cancer mortality and intake of certain non-lipid constituents of the diet including animal proteins (5,7,8). Epidemiological studies revealed that the incidence of breast cancer was higher as protein consumption increased (9). The association with animal protein was found to be stronger than with total protein (8).

Strong evidence that dietary factors, predominantly fat (10,11), can influence carcinogenesis has been derived from animal studies, but those studies have not provided definite evidence supporting a causative association between dietary protein and carcinogenesis (7,12). The effect of type of dietary protein on mammary carcinogenesis is controversial as well. Increases of MCA- (13) and DMBA-induced (14) tumor incidence, a reduction of DMBA-induced mammary tumors (15), or no significant difference in tumor yields between rats fed semipurified diets containing either casein or soy protein (16) have been reported. A diet containing 50% raw soybean has been reported to inhibit mammary cancer induced by X-irradiation in Sprague Dawley rats, which may be due to non-specific effects of protease inhibitors in the soybeans, as can be deduced from the reported poor growth rate of animals fed a soybean diet (17). It was found that high-protein diets increase the incidence of spontaneously occurring mammary tumors in rats (18,19), but not in mice (17,20,21) when fed diets with varying (9-45%) levels of protein. Wistar rats fed diets extremely high in casein (77%) and not treated with a chemical carcinogen gained less weight and developed fewer spontaneous tumors, including breast fibroadenomas, than animals fed 15% casein (22). A protein-rich diet (31% casein) enhanced in rats DMBA-induced mammary cancer incidence as compared with isoenergetic diets normal (19.5% casein) and low (8% casein) in protein, containing 10% corn oil as source of fat (14,23). In the DMBA-treated animals protein levels did not affect tumor multiplicity and latency (14,23). Different amounts of casein, fed during the promotional stage of DMBA-induced mammary carcinogenesis, had no apparent effect (15). Initiation of tumors, however, seemed to occur more readily in rats fed a low-protein diet (15). This is in defiance of what one

might expect from the positive correlation between animal protein and risk of breast cancer in humans as derived from epidemiological data (5).

Mammary tumor growth in experimental animals is affected by a number of hormones, in particular Prl, E_2, Prog and GH (24-26). Many studies on diet and mammary carcinogenesis have used rats exposed to chemical carcinogens such as DMBA and NMU. Most of the resulting tumors exhibited a high degree of hormone dependence, and regressed after ovariectomy (27), after hypophysectomy (28) or during treatment with antiestrogens (29). In addition, diet is known to affect endocrine status as well.

The present study was designed to investigate systematically the possible interactive effect of content and type of dietary protein (animal or vegetable) on the endocrine system in affecting NMU-induced mammary carcinogenesis at a constant level of fat (17.5% by weight). Plasma Prl and E_2 levels were assessed in NMU-induced tumor-bearing animals. In addition, ER and PR levels in selected tumors were also estimated simultaneously in the same sample. The NMU-induced mammary tumor model (30) was selected because NMU is an established carcinogen that can be administered systemically, avoiding possible differences in intestinal uptake and metabolism between groups fed different diets, which is likely with orally administered DMBA. Moreover, NMU-induced tumors exhibit histologic and endocrine features which closely resemble human breast cancer (31-33). The 17.5% fat level was selected as a moderate level from previous studies (10,11)

MATERIALS AND METHODS

Chemicals. NMU was bought from Ash Stevens, USA (ASI-701; PO#5496). [2,4,6,7,16,17-^{3}H]-17β-E_2 (s.a 160 Ci/mmol; NET-517), [17α-methyl-^{3}H]-trienolone (=R1881; s.a. 87 Ci/mmol; NET-590), and radioinert R1881 were obtained from New England Nuclear (Du Pont, 's-Hertogenbosch) Netherlands. [^{3}H]Org2058 (s.a. 45 Ci/mmol;TRK.629) and radioinert Org2058 were obtained from Amersham, Utrecht, Netherlands. DES (D4628) and Cc (C2505) were obtained from Sigma, St.Louis, MO. All tracers were stored in absolute ethanol at -20°C, and their purity was checked by chromatography. Antisera used were as reported elsewhere (10). All other chemicals were of analytical grade. Cristalline NMU was dissolved in a citric acid-phosphate buffer according to McIlvaine (36).

Experimental design, animals, animal care and induction of mammary tumors. For reasons of breeding, animal care and treatment the study was subdivided into two similar parts, I and II. Weanling animals, 21-23 days old were purchased from CPB-TNO (Zeist, Netherlands), and delivered in two different shippings. On arrival, twice 240 female Fischer F344 rats each were randomly assigned to fifteen dietary treatments arranged in a 3x5 (protein content by protein type) factorial design, as presented in Table 1. Groups consisted of 16 animals each and were housed 4 per cage. Dietary protein was fed at 15%, 22.5% or 30% by weight (LP, MP and HP respectively) at a constant level of

dietary fat of 17.5% by weight. Increased protein content of the diet was compensated by a decreased contribution of carbohydrates. At each of the three mentioned levels of protein, 5 different diets were composed such that the protein offered was either totally of animal origin, totally of vegetable origin, or a mixture of animal and vegetable protein (in ratios of 80/20, 50/50, or 20/80) to mimic protein contribution in totally carnivorous and totally vegetarian diets and in some intermediate ratios.

Table 1
Experimental design of a factorial study on the interactive effects of content and type of protein on mammary carcinogenesis in F344 rats by NMU.[a]

protein content		Casein:soy protein ratio ——>				
	%, w/w	1:0	4:1	1:1	1:4	0:1
LP	15	A	B	C	D	E
MP	22.5	F	G	H	I	J
HP	30	K	L	M	N	O

[a]16 animals per group; assay carried out in duplo.

Table 2
Composition of the diets at three levels of protein[a]

Ingredients	15 %	22.5 %	30 % (w/w)
Casein[b]	8.49	12.73	16.97
Soy protein isolate[b]	8.49	12.73	16.97
Wheat starch	27.50	23.30	19.06
Sucrose	27.50	23.30	19.06
Safflower oil[c]	2.10	2.10	2.10
Beef tallow[c]	15.40	15.40	15.40
Cellulose	5.80	5.80	5.80
Mineral mixture	4.06	4.06	4.06
Vitamin ADEK mixture	0.35	0.35	0.35
Vitamin B mixture	0.23	0.23	0.23
	99.92	100.00	100.00
Calculated energy	445.50	445.74	445.74 cal/100g

[a]Sources as reported previously (10)
[b]Based on the protein content of casein and soy-protein isolate, 89.8% and 87.8% respectively.
[c]Beef tallow and safflower oil together are responsible for 2.4% (w/w) linoleic acid to meet the linoleic acid requirement

Kept under well controlled conditions, the semipurified diets were fed ad libitum, from weaning until termination of the study. The diets were isoenergetically (445 kcal/100 g food), calculated with 4.0 kcal/g for protein and carbohydrate and 9.0 kcal/g for fat. Body weight gain and food intake were recorded weekly. Further details about animals and animal care were as reported previously (10). Mammary tumors were induced as in earlier studies (10) except that NMU was purchased from Ash Stevens. From 5 weeks after injection of NMU all animals were palpated once weekly, and the location and size of each tumor were recorded until the end of the study.

Composition of the diets. The diets were prepared according to principles outlined by Newberne et al. (35). Assuming that rats tend to consume equal amounts of energy when fed diets with different energy density, resulting in quantitative differences in food intake, the diets were composed such that equal intake of non-variable nutrients was assured. Casein and soy protein were used to achieve the desired protein levels in the diets. Safflower oil and beef tallow were used as sources of fat. Table 2 presents the composition of the diets and Table 3 the amino acid composition of the stock casein and soy protein isolate. All dietary ingredients were obtained from commercial sources and the diets were prepared in house. Every 4 weeks 5-kg lots were prepared and stored at +4°C in the dark until used. Quality control of the diets was performed as previously reported (10). Food was offered in powdered form in feeders of a design that prevents scattering. The food in the cages was changed completely twice a week. Food consumption was recorded to calculate energy intake and to observe food consumption patterns.

Termination of the study. Twenty-six weeks after administration of NMU, animals were sacrificed by decapitation with a guillotine (Harvard Bioscience, South Natick, MA, USA) in a quiet room and without ether anesthesia to avoid excitement of the animals, as described elsewhere (36). Blood was collected from the trunc in heparinized tubes (5000 IU/ml, Kabi, Stockholm, Sweden) and plasma was separated from it by centrifugation with Sure-sep (General Diagnostics, Morris Plains, NJ, USA) in a cooled centrifuge at 1200*g for 10 minutes. Plasma was frozen and stored at -20°C until assayed. A vaginal smear was taken within half an hour after decapitation to relate hormonal values to the stage of the estrus cycle.

Autopsy. Animals were sacrificed and autopsied either at the end of the study or in case of a moribund condition during the study, and inspected for gross tumors. All palpable and non-palpable mammary tumors were excised from the animals measured with a caliper and weighed. Tumors weighing over 1 gram were cut into pieces. A representative part of the tumor was saved for histopathology. The other part was frozen in liquid nitrogen and stored, at -70°C for biochemical determinations. The tumor tissues were examined by a pathologist after staining with hematoxilin and eosin. Each tumor was classified according to histological criteria previously described (37).

Plasma hormone measurements and hormone receptor estimations. Plasma hormone levels of Prl and E_2 were estimated by radio-immunoassay procedures as previously described (36). Content and affinity of cytoplasmic 'free' ER and PR were measured in tumors according to procedures reported in a previous publication (11).

Statistical analysis. Body weight, growth of the animals expressed as body weight gain, food intake, and food conversion efficiency were analyzed by analysis of variance (ANOVA), taking into account protein content, type of protein and their interaction, and adjusted for parts I and II of the study. Tumor incidence data (end-point analysis), tumor multiplicity (number of carcinomas per carcinoma-bearing animal) and tumor latency (time to first tumor appearance) were analyzed by Generalized Linear Models (GLM; 38), containing parameters for effects of protein content, type of protein, their interaction

and the stage of the estrus cycle at induction. The link functions going with these GLMs were logit, log and identity respectively, and the error distributions were binomial, poisson and normal respectively. Distribution of tumors over the different mammary pads was analyzed by χ^2 analysis. The plasma hormones Prl and E_2 were, after log transformation, analysed by regression analysis taking into account protein content, type of protein and their interaction, and the tumor-bearing status, the stage of the estrus cycle at termination of the study and their interaction. The ER, PR and their respective affinity constants were analyzed by regression analysis taking into account the same factors as for the plasma hormones, except the terms with the tumor-bearing status. In all analyses data were adjusted for parts I and II of the study. In all inorthogonal analyses all main effects were tested adjusted for all other main effects and all two-factor interactions adjusted for all main effects, but not necessarily for all other two-factor interactions.

Table 3
Amino acid composition of casein and soy protein isolate as estimated by absolute N measurement according to Kjeldahl.

	casein (N=14.3%)		soy-protein isolate (N=13.6%)	
Essential amino acids	g/100g	g/16gN	g/100g	g/16gN
Lysine	7.30	8.2	5.39	6.3
Methionine	2.66	3.0	1.12	1.32
Cystine	0.40	0.44	1.00	1.18
Threonine	4.03	4.5	3.22	3.8
Isoleucine	4.87	5.4	4.18	4.9
Leucine	9.04	10.1	7.21	8.5
Valine	6.34	7.1	4.34	5.1
Phenylalanine	4.65	5.2	4.48	5.3
Tyrosine	5.17	5.8	3.32	3.9
Tryptophan	1.15	1.29	1.15	1.35
Other amino acids				
Arginine	3.40	3.8	6.50	7.6
Histidine	2.68	3.0	2.19	2.6
Alanine	2.78	3.1	3.57	4.2
Aspartic acid	6.36	7.1	9.39	11.0
Glutamic acid	20.91	23.4	16.43	19.3
Glycine	1.70	1.9	3.44	4.0
Proline	10.70	12.0	4.65	5.5
Serine	5.76	6.4	4.78	5.6

RESULTS.

Growth, weight gain, food intake and food conversion efficiency. Animals fed LP, MP and HP diets had similar mean body weights at the start of the study. Considering body weight data grouped as to content of dietary protein fed during the first 77 days of the study more in detail, it appeared that animals

fed MP or HP had essentially comparable growth curves, as can be seen in Figure 1. Animals fed LP had significantly lower body weights (7.65 g; 3.95%; P<0.001) as compared with MP and LP. However, if grouped according to type of protein, animals fed vegetable/animal protein in ratios 1:1 and 1:4 had almost equal body weights. These were slightly higher than those of animals fed vegetable/animal protein in ratios 4:1 and 0:1, and markedly higher than those of animals fed vegetable protein only (data not shown). Body weight gain of animals fed LP was initially about 7% lower than of MP and HP fed animals. However, this effect disappeared in the course of the study. Animals fed vegetable protein only had initially a markedly lower weight gain than all other groups. This effect disappeared as well in the course of the study.

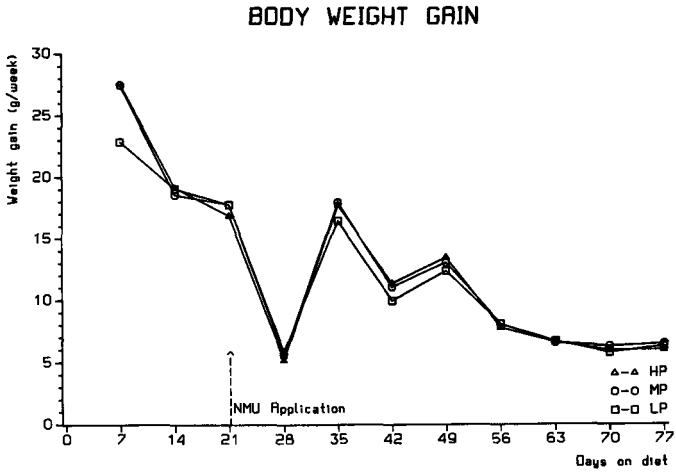

Fig. 1. Body weight gain of the animals during the first 77 days of the study. Arrow: NMU injection. Number of animals is 32. Data points indicate mean body weights, and SEM did not exceed 2.5% at any point. LP=low protein, 15% (w/w); MP=mid protein, 22.5% (w/w); HP=high protein, 30% (w/w).

Animals fed MP and HP isoenergetic diets consumed comparable quantities of food, 10.39 g/day and 10.03 g/day, respectively (equalling 46.3 and 44.7 kcal), while animals fed LP consumed 10.85 g/day (equalling 48.3 kcal) during the first 77 days of the study indicating that the LP group consumed most energy. This effect was only apparent during the beginning of the study and disappeared later. Food intake decreased immediately after NMU administration (Figure 2). Food intake data grouped according to type of protein demonstrated only a slightly increased intake in the 100% vegetable protein groups, but all differences between groups were at most 3.5% and disappeared during the course

of the study. Food conversion efficiency (FCE) was initially proportional to amount of protein in the diet. Animals fed HP had the highest FCE, followed by MP and LP. This relation remained during the initial phase of the study but disappeared after 8 weeks on the experimental diets. Related to the type of protein, there was a slight correlation initially, which vanished later on.

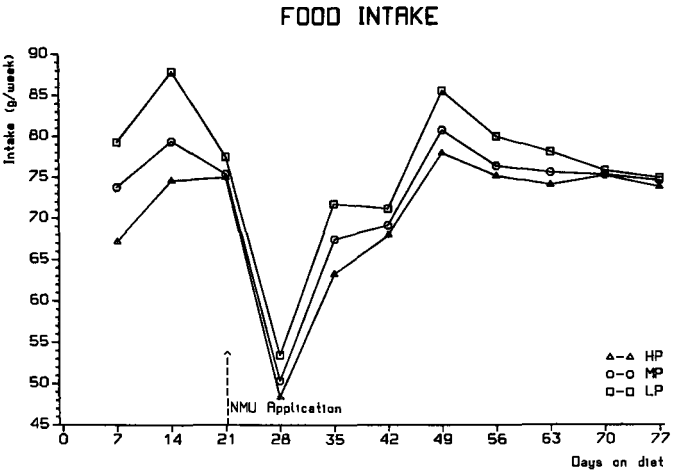

Fig. 2. Food intake of the animals during the first 77 days of the study. Arrow: NMU injection. Number of animals is 32. Data points indicate mean food intake, and SEM did not exceed 2.0% at any point. LP=low protein, 15% (w/w); MP=mid protein, 22.5% (w/w); HP=high protein, 30% (w/w).

Tumor incidence, tumor multiplicity and tumor latency. Statistical analysis revealed that final tumor development was significantly related to the stage of the estrus cycle at NMU exposure (Table 4). Rats in metestrus-diestrus ($P<0.01$), diestrus ($P<0.05$) or diestrus-proestrus ($P<0.001$) at that time were significantly less prone to develop tumors than animals in estrus. The effects of the various diets on mammary tumor development 26 weeks after NMU administration are summarized in Table 5. This table summarizes raw data grouped according to diet. They do not present a clear picture of possible effects of dietary treatments and they are strongly biased by estrus cycle influences. To avoid this bias, regression analysis was selected as the statistical method of choice. In subsequent tables results of this type of analysis are presented. It should be noted that analyses have been performed on log transformed data to cope with skewed distribution. As a consequence of having more effects than those presented, apart from having done the analysis sometimes on transformed

scale, presentation of standard deviations of the raw data in these tables is meaningless. Therefore they are not presented in the tables.

Table 4
Stage of the estrus cycle at NMU exposure in relation to tumor development[1]

animals	Stage of the estrus cycle[1]								total
	E	EM	M	MD	D	DP	P	PE	
total number	110	46	21	62	49	80	59	40	467
with tumors	4	5	3	11[a]	7[b]	14[c]	4	3	51
without tumors	106	41	18	51	42	66	55	37	416

[1] E=estrus; EM=estrus-metestrus; M=metestrus; MD=metestrus-diestrus; D=diestrus; DP=diestrus-proestrus; P=proestrus; PE=proestrus-estrus.

[a] significantly different from estrus, $P<0.01$
[b] significantly different from estrus, $P<0.05$
[c] significantly different from estrus, $P<0.001$

Table 5
Summary of experimental groups and their final tumor data

Group	Incidence[a]		Adenocarcinomas[b]		Multiplicity[c]		Latency[d]	
	I[e]	II[e]	I[e]	II[e]	I[e]	II[e]	I[e]	II[e]
A	13/16	16/16	49	53	3.76±0.54	3.31±0.44	78.2± 8.8	62.5±4.6
B	14/16	16/16	41	54	2.92±0.52	3.31±0.70	90.7±10.8	68.6±5.7
C	12/15	16/16	24	43	1.83±0.30	2.68±0.40	74.3± 7.1	77.5±8.8
D	11/16	15/16	29	48	2.63±0.60	3.20±0.36	116.6±15.3	80.8±6.5
E	14/16	14/16	30	32	2.14±0.29	2.28±0.35	96.5±10.0	83.5±8.4
F	12/14	12/15	43	33	3.58±0.46	2.75±0.44	72.5± 7.4	68.1±7.1
G	15/16	12/15	63	33	4.20±0.39	2.75±0.52	76.4± 3.5	77.2±9.1
H	15/15	14/16	51	43	3.40±0.39	3.07±0.34	92.8±11.1	69.6±6.6
I	16/16	12/16	40	46	2.50±0.30	3.83±0.35	95.9± 9.7	60.4±4.3
J	13/15	12/16	37	32	2.84±0.41	2.67±0.47	84.5±12.5	77.3±7.5
K	15/16	15/16	38	52	2.53±0.42	3.46±0.64	88.7± 6.7	68.3±5.6
L	15/16	14/15	49	48	3.27±0.55	3.43±0.44	87.3± 9.5	66.3±6.2
M	13/15	14/15	43	42	3.30±0.50	3.00±0.52	83.2± 7.8	77.8±7.3
N	13/16	16/16	38	46	2.92±0.46	2.88±0.33	102.1± 9.4	79.9±8.5
O	14/16	15/16	32	36	2.28±0.43	2.40±0.32	90.1± 9.0	78.2±5.4

[a] Incidence = number of tumor bearing animals/ number of animals per group.
[b] Adenocarcinomas = number of histologically verified adenocarcinomas.
[c] Multiplicity = number of adenocarcinomas per carcinoma-bearing animal ± SEM.
[d] Latency = mean time to first tumor appearance, in days ± SEM.
[e] I = first part of the study; II = second part of the study.

Analysis revealed that tumor multiplicity was dependent on the stage of the estrus cycle at induction ($P<0.001$) and on the type of protein ($P<0.005$). With increasing vegetable protein and decreasing animal protein, tumor multiplicity decreased during all stages of the estrus cycle, as can be seen in Table 5.1. These data are adjusted for the protein content, possible interactions between content and type of protein, and for part of the study (I and II). Neither effects of protein content nor interactions between content and type of protein on tumor multiplicity could be detected. Tumor latency was significantly influenced by the stage of the estrus cycle at induction ($P<0.001$) and by the type of protein ($P<0.01$; Table 5.2). Tumor latency was increased with increasing (up to 80%) contribution of vegetable protein. Remarkably, diets with 100% protein of vegetable origin yielded a shorter latency than the 80% group. Additionally, it appeared that mean tumor latency was significantly shorter in part II of the study than in part I ($P<0.001$; 17 days). This certainly points to the complexity of comparing animal studies. Table 5.2 presents the mean predicted tumor latency for diets varying in vegetable protein. These data are adjusted for protein content, possible interactions between content and type of protein, and for part of the study.

Tumor sites. Mammary tumors were not randomly distributed over the different mammary gland regions. Tumors originated in the right cervical and thoracic region (27.4%), the left cervical and thoracic region (33.1%), left abdominal and inguinal region (18.75%) and right abdominal and inguinal region (20.75%).

Table 5.1

Number of tumors per tumor-bearing animal grouped according to stage of the estrus cycle at NMU exposure and to vegetable protein contribution to the diet. Data are adjusted for protein content, possible interactions between content and type of protein, and for effects of part I and II of the study.

Vegetable protein %	Stage of the estrus cycle[1]							
	E	EM	M	MD	D	DP	P	PE
0	3.47	3.51	2.14	2.87	3.33	2.69	4.31	3.14
20	3.39	3.43	2.06	2.79	3.25	2.62	4.24	3.06
50	3.15	3.19	1.82	2.55	3.01	2.38	3.99	2.82
80	2.96	3.01	1.63	2.36	2.83	2.19	3.81	2.63
100	2.54	2.58	1.21	1.94	2.40	1.77	3.39	2.21

[1] E=estrus; EM=estrus-metestrus; M=metestrus; MD=metestrus-diestrus; D=diestrus; DP=diestrus-proestrus; P=proestrus; PE=proestrus-estrus.

Table 5.2

Mean tumor latency (in days), grouped according to stage of the estrus cycle at NMU-exposure and to vegetable protein contribution to the diet. Data are adjusted for protein content, possible interactions between content and type of protein, for effects of part I and II of the study, and for the stage of the estrus cycle at the time of exposure to NMU.

Vegetable protein %	Stage of the estrus cycle[1]							
	E	EM	M	MD	D	DP	P	PE
0	67.7	72.4	87.4	84.3	71.4	77.7	60.5	67.4
20	74.0	78.7	93.7	90.7	77.7	84.0	66.9	73.7
50	73.7	78.5	93.5	90.4	77.5	83.7	66.6	73.5
80	86.1	90.8	105.8	102.7	89.8	96.1	78.9	85.8
100	81.2	85.9	100.9	97.8	84.9	91.2	74.0	80.9

[1] E=estrus; EM=estrus-metestrus; M=metestrus; MD=metestrus-diestrus; D=diestrus; DP=diestrus-proestrus; P=proestrus; PE=proestrus-estrus.

Pathology. Data on tumor subclassifications, evaluated according to histopathologic criteria, are presented in Table 6. Most mammary tumors were classified as adenocarcinomas of the tubulo-papillary type (80%). Only a limited number of fibroadenomas was observed spread over all dietary groups. In the first week after NMU treatment 8 animals died while in the course of the study, 42/480 animals were sacrificed and autopsied mainly due to a moribund condition or because an excessive size of a tumor became inconvenient to the animal.

Plasma hormone levels. In Tables 7 and 8 mean plasma levels of Prl and E_2 for various protein contents are summarized, grouped as to stage of the estrus cycle at termination of the study and as to type of protein. The levels of plasma Prl were dependent on the stage of the estrus cycle at termination of the study ($P<0.001$) and were also influenced by the type of protein ($0.1<P<0.05$). Prl levels in animals fed either 100% animal protein or 100% vegetable protein are lower than in animals fed any of the mixtures as can be read from Table 7. Additionally, a slight increase of plasma Prl values was observed in animals with multiple tumors. Plasma levels of E_2 reflect their normal cyclic pattern. Neither content nor type of protein affected plasma levels of E_2; only a significant interaction between content and type of protein on plasma levels of E_2 was observed ($P=0.01$). As can be seen from Table 8.1 this interaction is not unambiguous.

Table 6
Mammary tumors induced by NMU in female F344 rats[a,b]

Type of tumor	Tumors			
	I	II	I	II
ADENOCARCINOMA	N	N	%	%
tubulo-pappillary adenocarcinoma (AT)	452	559	74.5	87.2
tubulo-pappillary adenocarcinoma, regressive (ATR)	43	47	7.0	7.3
cribriform-comedo carcinoma (CR)	43	6	7.0	0.9
compact-tubular adenocarcinoma (COM)	43	21	7.0	3.3
anaplastic (ANA)	3	2	0.5	0.3
FIBROADENOMA (FA)	23	6	3.8	0.9
	607	641		

Total number of animals:	470	I:234	II:236
Number of animals with mammary tumors:	418	I:205	II:213

[a] observed over all dietary groups.
[b] I = first part of the study; II = second part of the study.

Table 7

Mean Prolactin levels (in ng/ml) grouped according to stage of the estrus cycle and to vegetable protein contribution to the diet. Data are adjusted for the protein content, for possible interactions between content and type of protein and for effects of part I and II of the study. Data are expressed as the antilogarithms of the fitted means derived from regression analysis.

Vegetable protein %	Stage of the estrus cycle[1]							
	E	EM	M	MD	D	DP	P	PE
0	4.5	2.2	2.5	2.2	1.3	3.7	3.9	3.4
20	5.7	2.8	3.2	2.7	1.7	4.7	4.9	4.4
50	5.8	2.8	3.3	2.8	1.7	4.8	5.0	4.5
80	5.9	2.9	3.3	2.8	1.7	4.8	5.1	4.5
100	4.9	2.4	2.7	2.3	1.4	4.0	4.2	3.7

[1] E=estrus; EM=estrus-metestrus; M=metestrus; MD=metestrus-diestrus; D=diestrus; DP=diestrus-proestrus; P=proestrus; PE=proestrus-estrus.

Steroid hormone receptor levels. Groups A, K, E and O were selected for the assessment of steroid hormone receptor levels. Tumors of animals fed casein had significantly higher mean ER ($P<0.05$) and PR ($P<0.05$) contents than animals fed soy protein isolate; 43.6 (n=18) and 37.2 fmol/mg protein (n=13) respectively for ER, and 385 (n=20) and 278 fmol/mg protein (n=13) Aespectively for PR. The amount of protein in the diet influenced the affinity

constant of ER (LP: Kd=0.23 nMol/l; HP: Kd=0.19 nMol/l) but not the total number of binding sites. The type of protein also affected ($P<0.05$) the affinity constant of PR (casein: Kd=0.95; soy protein isolate: Kd=0.56 nMol/l).

Table 8

Mean estradiol levels (in pg/ml) grouped according to stage of the estrus cycle and to vegetable protein contribution to the diet. Data are adjusted for protein content, for possible interactions between amount and type of protein and for effects of part I and II of the study.

Vegetable protein %	Stage of the estrus cycle[1]							
	E	EM	M	MD	D	DP	P	PE
0	8.86	13.74	5.73	7.42	6.11	9.42	28.34	25.64
20	9.24	14.11	6.11	7.79	6.48	9.79	28.72	26.01
50	6.56	11.43	3.43	5.11	3.81	7.11	26.04	23.33
80	8.51	13.38	5.38	7.06	5.76	9.06	27.91	25.28
100	6.86	11.73	3.74	5.41	4.11	7.42	26.34	23.64

[1] E=estrus; EM=estrus-metestrus; M=metestrus; MD=metestrus-diestrus; D=diestrus; DP=diestrus-proestrus; P=proestrus; PE=proestrus-estrus.

Table 8.1

Mean estradiol levels (in pg/ml) grouped according to protein content, and to vegetable protein contribution to the diet. Data are adjusted for the stage of the estrus cycle and for effects of part I and II of the study.

Protein content	% Vegetable protein				
	0	20	50	80	100
LP	13.44	10.04	7.98	15.22	10.91
MP	9.72	13.69	11.98	9.61	9.83
HP	12.32	13.04	8.82	9.61	8.85

DISCUSSION

The factorial design of our study, the careful registration of food intake and the application of advanced statistical methods provided tools to evaluate individual effects of amount and type of protein as well as their interaction on NMU-induced mammary carcinogenesis. In contrast to data on dietary fat (10, 11), those on dietary protein and mammary carcinogenesis are not consistent

(14-17,20,23). This study clearly shows that rats fed protein levels of 22.5% or 30% at a constant level of fat (17.5%) and treated with NMU had comparable growth patterns. However, animals fed LP showed lower body weights than animals fed MP or HP. In addition, animals fed vegetable protein only demonstrated a delayed growth pattern. Moreover, our results indicate a clearly toxic effect of NMU on food intake behavior, as expressed by a dramatically decreased food intake (Figure 2), a lower body weight gain and a pronounced decrease of FCE in the week after NMU application. Similar results have been reported by Nakagawa et al. (18) with protein fed at different levels ranging 10-36% (at 10% corn oil). Growth rate varied directly with protein intake early in the experimental period, but eventually the differences observed among these groups became insignificant (18). Recently, Sanz et al. (39) reported that rats fed isoenergetic LP diets (8% casein; 10% corn oil) showed delayed sexual maturation, although they consumed more food per 100 g body weight than rats fed HP (31%) diets, as is in line with our findings for LP fed animals. This delay was accompanied by both growth retardation and a retarded differentiation of the ducts in the mammary gland, indicating that such a LP diet may have a direct and specific effect on the growth of mammary epithelium. This hypothesis is supported by Rosso et al. (40), who demonstrated that a 50% food restriction during gestation restricted cell division and reduced the number of parenchymal cells in the mammary gland. Together, these results confirm that both dietary protein and dietary energy restriction may influence the growth and morphology of the mammary gland. This is likely to affect carcinogenesis, as well. Earlier studies of Hawrylewicz et al. (14) support this hypothesis. LP diets (8% casein, 10% corn oil) delayed sexual maturation dramatically: expressed as day of vaginal opening, this group averaged 68.6 ± 3.6 days, versus 37.0 ± 0.45 and 30.5 ± 0.3 days for the 19.5 and 31% casein groups. Thus, one may conclude that restriction of dietary protein may clearly reduce growth when the restriction is severe and that it also influences mammary gland development and ovarian function. One may speculate that under such circumstances even initiation of mammary carcinogenesis may be delayed or prevented.

Our results do not support any significant effect of the level of dietary protein on NMU-induced mammary tumor incidence. This is unlike results of Hawrylewicz et al. (14) who have reported a significantly increased tumor incidence from 58%, 76% to 100% in SD rats. However, they used DMBA as the

carcinogen and applied a relatively low-fat (10% corn oil) level in their rations. However, their results were only significantly different for the groups with 8 and 31% protein. Additionally, they studied the influence of animal protein only. The dose of carcinogen as used in our study (although generally accepted: 50 mg/kg body weight) may have been too high to discriminate possible dietary protein influences. As discussed previously (10), we did not analyze tumor count data because they are not indicative of dietary involvement in mammary carcinogenesis per se.

Tumor multiplicity and latency were not influenced by the content, but only by the type (vegetable) of dietary protein. Recently, Park et al. (41) reported that the type of dietary protein can influence DMBA-induced mammary carcinogenesis, whether energy intake was normal or reduced. They compared isocaloric diets, containing 18% of casein, soy protein, cottonseed flour, wheat gluten, and casein pair-fed to wheat gluten. At ten weeks after DMBA administration the incidence was 40%, 80%, 47%, 27%, and 13% respectively. Dietary intakes of casein, soy protein and cottonseed flour were similar but significantly higher than those of the wheat gluten or pair-fed groups. These different tumor incidences indicate effects of type of protein, but one should realize that their observation was done only 10 weeks after DMBA application, which is considerably less than about half the time most studies last. Furthermore,their report lacks information on the fat level of their diets.

Data on the influence of the type of protein on mammary carcinogenesis in rats (13,15-17) and mice (20) are to date contradictory and have been incompletely reported. Feeding a relatively lysine- or cysteine-deficient diet, diminishes the outgrowth of mammary tumors in mice (42,43). Mice fed LP, low-cysteine diets show disturbed estrus cycles (42). However, these early studies have not been performed according to current standards for animal nutrition studies (35). From adequately performed studies it has been concluded that suppletion with lysine do not affect mammary tumors in mice (20). From studies on chemically induced rat mammary carcinomas (44) and spontaneous mammary tumors in mice (20) it appeared that both ornithine and tryptophane had no modifying influence. However, diets supplemented with arginin (45) or methionine (46) appeared to decrease the incidence of DMBA-induced rat mammary tumors. Methionine data, unfortunately, are not well documented (46), while arginine data (45) are reliable and can be reproduced consistently (44). Recently, Burns et al. (47) showed that supplementing a 14% casein diet with 5% L-arginine decreased tumorigenicity (incidence and multiplicity) in both

NMU- and DMBA-induced tumors, suggesting specific inhibitory effects of arginine on stages of the chemical carcinogenesis other than bioactivation of procarcinogens. It is quite possible that the observed inhibitory effect of arginine results from arginine-induced alterations in hormone levels of Prl and GH (48).

Our data indicate that neither content nor type of protein affects plasma levels of E_2. Although a significant interaction between content and type of protein on plasma levels of E_2 is observed (P=0.01), this interaction is not unambiguous and cannot yet readily be explaned. Besides the expected cyclic pattern, plasma Prl was markedly influenced by the type of protein in the diet, suggesting that a mixture of animal and vegetable protein in the diet is likely to result in higher Prl levels in animals than either 100% animal or 100% vegetable protein. The slight increase of plasma Prl values observed in animals with multiple tumors may be ascribed to stress-inducing handling rather than to effects of dietary protein per se (36). Huang et al. (23) reported that animals fed 8% casein diets had significantly lower levels of circulating Prl, E_2 and Prg at every stage of the estrus cycle than groups fed 19.5 or 31% protein at 13 weeks of age. But in the course of their study these effects disappeared. Studies including isocaloric diets containing casein and corn oil at 19% and 15% or 33% and 15% also failed to find any effect of dietary protein on levels of Prl, E_2 and GH (49), just like a study with diets using 20% fat (50). Our study is in line with these results.

Studies on the combined effects of dietary protein (casein) and fat (corn oil) did not show substantial influences on DMBA-induced tumor incidence (51) nor on serum Prl (52). However, corn oil increased tumor incidence irrespective of the source of dietary protein. Additionally, tumorigenesis increased in rats with a higher ad libitum food consumption (51). The protein content of the diet only contributed to a significant fat-by-protein interaction with the size of adenocarcinomas. Comparable studies observed significant positive associations of energy consumption, adjusted for protein and fat intake, with tumor incidence (53). Gridley et al. (54) reported that C3H mice, fed diets containing either 11% or 33% protein and either 5% or 30% fat, showed most pronounced variations in spontaneous tumor incidence for the high protein. Low-fat low-protein diets or diets high in fish protein exhibited the lowest tumor incidence (54). Both content of dietary fat and reduced energy intake can influence mammary carcinogenesis, as discussed in previous reports of our group (10,11) and others (55-57). Reduction of energy intake has been found to

consistently decrease the incidence of tumors, including spontaneous mammary tumors, in a wide variety of experimental models, and most studies were accompanied by reduced growth rate and lower final body weights (56,57).

Recently, the interactive effects of fat (10% and 15% corn oil) and protein (8%, 19.5% and 31% casein) on mammary carcinogenesis were studied in a two-generation design (58). Independently of NMU or DMBA, tumor burden correlated positively with dietary protein. Animals switched to a high-protein high-fat diet after NMU application showed an increased tumor incidence. Serum estrogen, Prl, Prg and GH were not influenced by dietary protein. In this study, however, both the dam and her female offspring were fed the same test diets. Data may hence reflect a prolongued limited dietary protein intake or changes in cancer susceptibility as a result of any influence on the developing endocrine system.

Some workers have tried to explain effects of dietary protein on mammary carcinogenesis by a changed metabolism of the carcinogen. Clinton et al. (15) studied hepatic AHH, an enzyme that converts DMBA into hydrophylic products. They found an increased activity of AHH, cytochrome P-450 and cytochrome c-reductase with increased protein intake for 4 weeks, from 7.5% and 15% to 45%, in the livers of rats prior to DMBA administration. In light of future tumor development their results partly support the hypothesis of changed carcinogen metabolism, but it is still not unequivocally clear that increasing dietary protein decreases the response to a carcinogen by stimulating its detoxification. In another study, using either 20% corn oil or 20% beef tallow or a 50/50% mixture (50), they found that cytochrome c-reductase and P-450 content were higher in rats fed corn oil or mixed fat than in rats fed tallow. However, AHH, glutathion transferase and UDPG were not influenced, so that the effect of lipid source on carcinogenesis may be attributed to an altered carcinogen-metabolizing enzyme activity. In a previous study we found that both content and type of protein significantly influenced hepatic phase I and phase II biotransformation enzyme activities in livers of rats bearing NMU-induced mammary tumors (59). In favor of increased detoxification processes is the finding that total metabolism of DMBA in isolated cells increased as dietary protein increased from 7-15% prior to DMBA administration (59).

Taking all studies together, we suggest that most studies that observed effects of diets low in protein and/or low to very low in fat on mammary carcinogenesis, have actually dealt with effects of limiting factors of the

diet on growth and maturation of the animals prior to or concomitant with carcinogen treatment. Feeding 8-15% protein at a concomitant level of 10-17.5% fat may approach a status of deficient to marginal nutrition leading to delayed development and delayed differentiation of mammary epithelium and ovarian function. The terminal end-buds are supposed to be the target tissue of mammary carcinogens (61). Therefore, it is likely that the stage of development of animals in case of deficient diets such as low-protein rations (possibly more pronounced for vegetable protein than for animal protein) does not meet the requirements of cellular differentiation and hormonal function needed for optimal expression of a carcinogen at the time of exposure at 50 days of age, as is usually the case in rat studies. If that holds true it is not of prime importance whether the carcinogen acts directly or indirectly but rather that the biological sensitivity of the tissue is the limiting factor.

It is concluded that dietary protein fed at levels of 15%, 22.5% or 30% (w/w) at a concomitantly high level of fat (17.5%) does not affect the incidence of NMU-induced mammary tumors although it must be recognized that a high dose of carcinogen as used in our study may disguise any effects. However, both amount and type of protein play a role in carcinogenesis. Substituting vegetable protein for animal protein results in lower tumor multiplicity and a longer latency period. Although plasma Prl and E_2 levels of animals fed 100% vegetable protein were lower than those of the other groups it is not likely that both of these hormones play a causative role in NMU-induced mammary carcinogenesis in F344 rats due to variations in dietary protein as studied here.

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CHAPTER 7

GENERAL DISCUSSION

Epidemiologic studies have revealed that breast cancer rates are significantly different between countries. The key difference in environmental factors between low risk countries such as Japan and high risk countries such as the United States and the western European countries (including Netherlands) is dietary, with the main variable being the quantity of dietary fat (1). In general, high risk populations consume a higher proportion of dietary fat than low risk populations (2). In contrast to the increase in risk of colon cancer occurring in first-generation migrants from a low risk region (Japan) to a high risk area (USA), it is not until the seond generation that a risk similar to that of longterm residents in the high-incidence region is attained. This aspect probably suggests that, for breast cancer, residence in a high risk area at the time of puberty and breast development is critical (3). Perhaps relevant to this concept is the observation that female Sprague Dawley rats apppear more sensitive to mammary carcinogens at or around puberty than older or younger animals and that this susceptibility seems to be correlated with serum prolactin levels and the rate of cell division (4). Living in the high risk area during puberty may be conducive for acquisition of a high risk.

The preceeding chapters of this thesis have dealt with a number of aspects relating to mammary gland tumorigenesis. The goals of the study were essentially two-fold:

1. to test the hypothesis that dietary fat and protein influence development of NMU-induced mammary carcinogenesis in rats, and
2. to assess whether these influences are mediated by altered hormone levels of prolactin, estradiol-17β, progesterone, corticosterone, and/or by altered steroid hormone receptor content in mammary tumor cytosol.

For obvious reasons animal studies have some advantages over human studies to assess relationships between diet, hormones and cancer. However, one should

realize that results from animal studies can not easily or directly be extrapolated to the human situation as a result of differences in dose level or route of administration of the carcinogen, composition of the diets, and species dependent differences in metabolism. Nevertheless, they may substantially contribute to the understanding of possible mechanisms. Postulated mechanisms by which dietary fat may influence mammary carcinogenesis include , a.o.:

- direct effects on host endocrine metabolism,
- secondary effects of obesity, due to high dietary fat
- suppression of immune function,
- inhibition of intercellular communication,
- formation of lipid peroxides and/or oxygen radicals,
- direct effects on the tumor cell,
- influence on prostaglandin synthesis and/or metabolism.

In this thesis we focussed mainly on the first mechanism mentioned. Dietary fat may elicit its tumor-enhancing effects by altering host endocrine metabolism, in particular, prolactin secretion. The interest in a fat-prolactin relationship in breast cancer is prompted by at least three facts:

a. prolactin can act as promotor in a number of murine breast tumor systems,
b. prolactin is a liporegulatory hormone in birds and lower mammals, rodents, and possibly humans, and
c. elevated prolactin levels have been observed in tumor-bearing animals.

Moreover, also other hormones, such as estrogens, GH, and prostaglandins are involved in growth control of the mammary gland. It is of interest to define the nutritionally-linked hormonal balances associated with mammary carcinogenesis.

DIETARY FAT AND HORMONES IN NON TUMOR-BEARING ANIMALS.

We primarily investigated the influence of amount and type of dietary fat in healthy, cyclic, female F344 rats. The results of a series of studies undertaken to obtain more detailed information on the influence of amount and type of dietary fat and the effect of ether anesthesia during bloodsampling on plasma levels of prolactin, estradiol, progesterone and corticosterone were

discussed in Chapter 3. It was shown that different levels of dietary fat from lard and sunflower seed oil, did not influence plasma levels of prolactin and estradiol. However, progesterone levels during proestrus, were higher in rats on high and lower in rats on low sunflower seed oil, both if compared with hormone levels of rats on high and low lard. Sunflower seed oil was associated with higher levels of corticosterone than lard, irrespective of the amount of fat and of the stage of the estrus cycle. Large effects of ether anesthesia were found in comparison to decapitation even after adaptation to the handling procedure. These effects varied during the different stages of the estrus cycle, and were particularly marked during met-diestrus. These results do suggest that differences in plasma levels of prolactin and some steroid hormones as reported in several publications are more likely due to insufficiently standardized experimental procedures, including stress-inducing handling and manipulation of the animals, than either to amount or type of fat in healthy rats. The data also stress the importance of confirmation of the stage of the estrus cycle by careful interpretation of vaginal smears in experimental use of female rats.

Stress seems to have a paradoxical effect on plasma levels of prolactin in female rats. In the morning it induces an increase and in the afternoon a suppression of prolactin and this is regardless of the method of stressing (5). In our study ether inhalation also induced an increase in plasma prolactin levels during the morning of metestrus-diestrus and not in the afternoon of proestrus. This is in agreement with the observation that high serum LH and prolactin levels in the afternoon of proestrus are not affected by ether vapor stress (6,7). Moreover, this ether-induced rise in plasma prolactin appears to be quit rapid. In male rats the response to a three minute ether stress is rapid but transient, with a peak at about ten minutes after the onset of stress (8). In females a tenfold rapid and transient increase in afternoon prolactin levels was observed in one study (8). The stress-induced response of prolactin in both handled and non-handled animals was similar but differed in magnitude (8), or lowered serum prolactin levels in female rats opposite to male rats (9). 'Handled' rats could have adapted somewhat and thus become less responsive to parts of the multiple stress used (9). Male Long Evans and Sprague Dawley rats exhibited increased but transient prolactin levels during serial sampling after decapitation and orbital sinus blood collection. Ether inhalation for 1 minute increased serum prolactin levels in both sexes of SD

rats with a peak value at 2.5 minutes after ether vapor exposure and returning to basal levels by 15 minutes. Handling of female rats for 4 days lowered serum prolactin levels but the same procedure raised prolactin levels in male rats (10). Short ether anesthesia did not alter serum levels of testosterone and FSH, but it increased serum LH and prolactin levels significantly and these reactions were not uniform, but rather strain and species dependent (11, 12).

Sofar it can be concluded that ether is able to induce shifts in prolactin secretion probably via a central neural mechanism. Prolactin secretion is regulated by hypothalamic nuclei including catecholamines (13,14). Stress caused elevated levels of pituitary cAMP and plasma B-endorphin (15). Handled animals may have an altered sensitivity to compounds with dopamine activity (16). In addition, opioid peptides can reverse the inhibitory effects of dopamine directly at the level of the pituitary gland (17), while prolactin can also stimulate dopamine activity (18). Several amino acids appeared to be potent stimuli for prolactin secretion (19-21). Thus both dietary factors and ether can influence the central prolactin release regulating mechanism. Uptake of 5-hydroxy-tryptamine is necessary for timing and/or magnitude of the spontaneous prolactin surge (22). In addition, thyrotropin releasing hormone (TRH) is involved in the release of prolactin from the anterior pituitary gland (23). Blocking of TRH delays the prolactin release. Unfortunately we did not measure TRH in our study.

Since the endocrine system is vulnerable to stressful events, the response to handling prior to decapitation alters hormonal levels and secondarily many other processes. This inadvertant stress challenge confounds the response of an animal to exogenous compounds. The potential influence of stress must be given adequate consideration in endocrine-related studies. Thus, studies of hormone levels in relation to mammary carcinogenesis require standardized procedures for collecting blood, which is used for hormone estimations and, for experimental manipulation of the animal prior to obtaining blood. Rats are macrosmates and perceive mainly olfactory stimuli. All sorts of irritation, including smelling of blood should be avoided to eliminate all signs of danger to the animals. A method of collecting blood in a way "poor of stress" from animals seems to be decapitation in a separate room.

Although it is increasingly evident that tumor development is a complex multistep process, the original two-stage 'initiation and promotion' model of carcinogenesis has proven useful in studies with laboratory animals to determine mechanisms by which dietary fat may influence experimental mammary carcinogenesis. In Chapter 4 we presented a systematic study to establish the possible interactive effects of percentage of LA and total amount of fat as dietary factors in breast tumor promotion. In contrast to most studies selecting high versus low doses of dietary factors in either two groups or in a 2x2 factorial design, we selected a 3x3 factorial design. Therefore we were able to compare low versus moderate and high contents of either dietary fat or LA in the diets. Using multivariate and regression analysis we were able to shown that increasing the content of either fat or LA did not markedly influence tumor incidence.

Increasing the LA content resulted apparently in an increased tumor multiplicity, although not statistically significant. Increasing the content of both fat and LA in the diet resulted in a non-significantly shortened time to first tumor appearance and a higher tumor yield. We concluded that a high LA content of the diet was associated with a higher mammary carcinoma yield, although this effect was not statistically significant. LA as such does not appear to affect mammary tumor incidence as strong as does total amount of fat in the diet. From our study there appears to be no treshold of LA with respect to its enhancing effect on mammary carcinogenesis for dietary levels between 0.85% and 5.31% w/w (1.70-12.53% of energy). This is partially in agreement with results of Ip et al. (24) who interpreted their results -in studies with high fat diets containing 20% coconut oil- that raising the LA content from 0.5-4.4% by the substitution of palm oil at the expense of coconut oil did not further increase tumor respons. Main differences between the two studies involve the use of different sources of dietary fat and LA, as well as the use of three levels of dietary fat in our study. Several studies provide evidence that the enhancing influence of dietary fat on mammary carcinogenesis is directly related to the proportion of polyunsaturated fat (25,26). Therefore, more studies are needed to further quantify this phenomenon.

In our study we also found strong evidence for the involvement of growth and development and for a role of the endocrine system in the process of carcinogenesis and more specifically at the initiating step. Rats that ultimately developed tumors grew faster in the last week before NMU application, than animals that did not develop tumors. A significant influence of the stage of the estrus cycle at the time of NMU-exposure was found as well. Rats in diestrus at the time of NMU-exposure were more prone to develop tumors than animals in the other stages of the estrus cycle at that time. This makes it likely that endocrine factors, establishing the hormonal milieu at the time of carcinogen exposure, play an important role in chemically induced rat mammary carcinogenesis. Perhaps by supporting NMU-DNA interaction and fixation of DNA damage, or by a certain influence on cell division activity.

FAT, LA AND HORMONES IN MAMMARY CARCINOGENESIS.

Interactive effects of dietary LA and total fat content, and plasma levels of prolactin, estradiol, progesterone and corticosterone as mediators in mammary carcinogenesis have been described in Chapter 4. In addition, ER, PR and AR levels in the tumors were estimated simultaneously in the same sample. It appeared that the level of fat, but not the LA content in the diet influenced the plasma levels of prolactin and estradiol. However, it was not a unique and clear one way effect. The effect of fat varied for different levels of LA. Plasma estradiol levels increased with increasing fat content of the diet from 10% to 17.5%, but decreased with further rise of the fat content to 25% at every LA content tested in our study design. A non-linear effect of LA, but no effect of fat on plasma progesterone was found. Plasma levels of corticosterone appeared not to be influenced by either dietary LA content or amount of dietary fat.

We did not find differences in hormone levels of tumor-bearing and non-tumor-bearing animals. This makes it clear that plasma levels of hormones are not a good parameter to study possible interrelationships between dietary fat or LA and chemically induced rat mammary carcinogenesis in a setting as used in our study where diets were adequately composed. It may well be possible that plasma hormone levels are determinants in some way in case of diets very low or deficient in fat, as is likely to happen in studies using 0.5% fat.

The level of fat in the diet did not influence content and affinity of ER, PR and AR in NMU-induced mammary tumors. Receptor content tended to decrease with increasing lifespan of tumors, perhaps reflecting a shift to hormone-independent cells. We were not able to discriminate between tumors on base of steroid hormone receptor data. Additionally, no correlations between plasma hormone level and corresponding hormone receptor content in the tumors were found in our studies.

ENERGY INTAKE AND MAMMARY CARCINOGENESIS.

Feeding high fat diets to rats generally produces an enhanced response of carcinogen induced tumors. However, there are inherent difficulties in attempting to address dietary fat per se as the factor responsible for the effects of dietary fat on mammary carcinogenesis. As the level of fat in the diet is increased, the energy density of the diet rises rapidly. The energy density of diets is usually modified by changing the ratio of fat to carbohydrate. In making such changes, it is not uncommon that other dietary constituents such as e.g. fiber, vitamins and minerals change as well. Therefore it is very difficult to implicate the manipulation of one specific nutrient in the diet as being unambiguously responsible for changes in tumor incidence. It has been suggested that enhancement of mammary tumor formation by dietary fat may be mediated by increased energy intake. Early studies by Tannenbaum (27) were the first to show that restriction of energy intake will decrease the incidence of spontaneous mammary tumors in mice. Animals subjected to chronic food restriction not only had fewer mammary tumors, but tumors also appeared later than in animals fed ad-libitum. Ross et al. (28) concluded from their studies with rats that the risk of a rat developing a spontaneous tumor is directly proportional to calorie intake and growth rate early in life; protein intake is also involved but in a more complex way. It is sufficiently known that adequate energy supply from fat, protein and carbohydrates is essential for normal growth, reproduction and overall physiological functioning. However, the overall requirements depend on several factors such as animal strain, species, age, sex, housing, ambient temperature and physical exercise. Standards for normal body weights, referring to these variables have not been defined.

In case of mammary carcinogenesis, it has been reported that a reduction of energy intake of 20% or more is associated with a significantly decreased tumor response. It has also been reported that fat calories are more efficiently utilized for growth relative to carbohydrate or protein calories (29), which is reflected in more energy retention in the carcass of the animal as body fat (30) and explains why body weights of animals fed high fat diets were higher in several studies (29-32). Our results show a roughly 8% higher energy intake and about 6% heavier body weights, compared to animals fed moderate fat and low fat diets which is in line with these observations. It also stresses that relatively small differences in body weight may reflect larger differences in body composition, which in turn may account for significantly different carcass energy. This issue needs further investigation to understand the influence of net energy on carcinogenesis. At the same time one must bear in mind, that caloric restriction per se induces a wide range of physiological changes, particularly in the endocrine and immune system, and perhaps diverse mechanisms mediate the inhibitory effects of a certain state of 'underfeeding' on carcinogenesis in general.

DIETARY PROTEIN AND MAMMARY CARCINOGENESIS.

In considering the less consistent and still poorly understood role of dietary protein, we studied the effects of casein (animal protein) and soy protein isolate (vegetable protein), at a relatively high constant level of fat (17.5%) in the diet in a 3x5 factorial design. Increasing the protein content at this level of fat, did not influence tumor incidence. The lowest number of tumors was observed in animals fed soy protein isolate only. As in our earlier study on LA and fat content of the diet, tumor development was related to the stage of the estrus cycle at the time of exposure to NMU. Tumor multiplicity was dependent on both the stage of the estrus cycle at induction and on the type of protein consumed. Increasing the proportion of vegetable protein at the expense of animal protein was accompanied by a decreased tumor multiplicity and by a prolongued latency. Dietary protein influenced plasma prolactin levels not unambiguously. Plasma prolactin levels from animals fed either 100% casein or 100% soy protein isolate were lower than those of any of the mixtures (80/20, 50/50, 20/80) tested. Plasma levels of estradiol-17β were unaffected by either type or amount of dietary protein. The tumors of animals fed casein had higher mean ER and PR receptor levels than animals fed soy

protein isolate, perhaps indicating that there is a limiting amino acid in soy protein isolate fed animals.

The growth rate of rats fed different protein diets varies directly with the protein intake in the early period, but eventually those differences disappear (33). Sanz et al. (34) and Hawrylewicz et al. (35) observed a delayed sexual maturation in rats fed isoenergetic low protein diets (8% casein; 10% corn oil). This delay was accompanied by both growth retardation and a retarded differentiation of the ducts in the mammary gland, indicating that such a low protein diet may have a direct and specific effect on the growth of mammary epithelium. Rosso et al. (36) support this hypothesis by results demonstrating that a 50% food restriction during gestation restricts cell division and reduces the number of parenchymal cells in the mammary gland. It is most likely that low protein diets are deficient of essential amino acids, leading to pituitary insufficiency, decreased secretion of anterior pituitary hormones and decreased estrogen production. Thus, severe restriction of dietary protein influences both mammary gland development and ovarian function, conditions which both interfere with initiation of mammary carcinogenesis.

Age is an important factor in determining the response of the mammary gland to various carcinogenic stimuli in rats. From studies of DMBA-induced mammary carcinogenesis, it became evident that rats are most susceptible to the effects of the carcinogen at the age of 30-55 days, closely around the time of sexual maturation, and beginning of the estrus cycle (35-42 days). At about 40-46 days of age the density of and the cellular proliferation in terminal end buds, supposed to be the target cells for chemical carcinogens, is at a high level (37). Exposure to DMBA at that time results in 100% tumor incidence, while exposure at older ages results in reduced numbers of mammary tumors. This aspect has not been studied by our group but this information is needed to further understand relationships. The developing mammary gland responds to systemic hormonal stimuli of prolactin, growth hormone, estrogens, progesterone and corticosteroids and perhaps thyroid hormone. Estrogens and prolactin induce mitosis in mammary epithelial cells. By promoting cellular replication and DNA synthesis, these hormones sensitize the mammary gland to carcinogen binding. As can be derived from the above discussed studies on dietary fat and dietary protein in relation to mammary tumors, diet can play an important role in chemical carcinogenesis. However, both anatomic structures which are susceptible to carcinogenic stimuli and a well developed functioning endocrine system are prerequisites. Deficient, marginal or

restricted diets will most likely result in decreased secretion of anterior pituitary and gonadal hormones. The hormonal deficiencies that develop around or directly after exposure to the carcinogen may thus be responsible for the inhibition of mammary tumor development.

Here we come to a very interesting point in the possible involvement of diet and hormones in cancer. Studies on diet and cancer may be biased by unwanted side effects. In studying the effects of dietary variables at least four options are available for administration of experimental diets,

- feeding experimental diets from weaning on, until termination of the study (combined phases),
- feeding standard formulations to all groups, and changing to the experimental diets directly after application of the carcinogen (promotion phase),
- feeding experimental diets prior to exposure, and switching to one standard formulation directly after after exposure (initiation phase), and
- two generation studies, where the offspring receives the same diets as the mother.

Diet directly influences the development of tissues, and a state of undernutrition leads to incompletely developed endocrine function. Therefore it may well be possible that the state of development and differentiation of the mammary gland in case of combined phase studies or initiation phase studies with low fat (0.5% corn oil) or low protein (8% casein) compared with high fat or high protein diets reflect a dissimilar starting point. Inhibiting effects as ascribed to diet, may actually reflect indirect effects on carcinogenesis. This again may explain why there is no satisfactory evidence for a direct cause-effect relationship between hormones and cancer. Hormones may play an indirect rather than a direct role in development of neoplasia and they act primarily by mechanisms still poorly defined, as promoting and permissive agents. In addition, hormone stimulated proliferation may increase the probability of errors in DNA replication, giving rise to genetically altered variants, which may propagate and eventually express the tumor phenotype.

GENERAL CONCLUSION:

The relationship between diet and mammary cancer is a very complex one. Diet directly influences the development of tissues, and therefore it can play an important role in chemical carcinogenesis. Diets, deficient or marginal in

essential fatty acids or essential amino acids, most likely lead to pituitary insufficiency, decreased secretion of anterior pituitary hormones and decreased estrogen production. At the same time growth will be retarded, including development of mammary epithelium. The developing mammary gland responds a.o. to systemic hormonal stimuli of prolactin and estrogens. Estrogens and prolactin induce mitosis in mammary epithelial cells. By promoting cellular replication and DNA synthesis, these hormones sensitize the mammary gland to carcinogen binding. The multistep process of carcinogenesis, including the reaction of a normal cell genome with a genotoxic agent may result in an irreversible change in DNA, generally referred to as initiation. As yet unknown stimuli (probably particular growth factors), however, may promote to proliferation of preneoplastic cells, a process which may take several substeps of which some are reversible. However, only under favorable conditions the steps from initiation to progression of preneoplastic cells may be taken succesfully. Thus, both sufficiently developed anatomic structures, which are susceptible to carcinogenic stimuli (most likely the teminal end-buds) and a well developed endocrine system are needed to initiate carcinogenesis. In successive tumor promotion, both hormones and diet (most evident dietary fat and energy consumption) have a certain stimulating role, but it is unlikely that dietary factors in adequately fed animals affect tumorigenesis through influences on plasma levels of steroid hormones or prolactin.

PERSPECTIVE

Some comments on possible mechanisms have already been made in the above discussions. It is evident that the relation between diet and mammary cancer is a very complex one in which more factors than only macro- or micronutrients are involved. The multistep process of carcinogenesis, including the reaction of a normal cell genome with a genotoxic agent may result in an irreversible change in DNA, generally referred to as initiation. Initiated or transformed cells may remain silent as preneoplastic cells for considerable periods of time. As yet unknown stimuli however, may promote the proliferation of preneoplastic cells into neoplastic cells, a process which may take several substeps of which some are reversible. In man numerous cells will be reached by very small quantities of potentially initiating carcinogens. Only under favorable conditions these compounds will be succesful in initiating, leading

to DNA damage and proliferation of preneoplastic cells. From both epidemiological and experimental studies it becomes probable that fat, caloric intake and may be alcohol (not discussed in our studies) seem to be powerful factors in the process of promotion. Yet unknown interactions with other factors, both stimulating and inhibiting, may be present. Inhibiting dietary factors such as fiber, β-carotene, vitamins A, C and E and a mineral as Se have not been discussed in the present studies, but they are believed to function through their antioxidant capacity. The extremely complex interactions between dietary factors and organ systems, such as the endocrine system and the immune system (not discussed in our studies), and their effects on cellular and subcellular systems including membranes and biochemical pathways, warrant further and detailed research in both experimental animals and man, to better elucidate the actual active mechanisms.

RECOMMENDATIONS TO THE PUBLIC ?

The awareness of the important role which diet plays in carcinogenesis has not yet brought us effective, preventive measures or strategies. One may wonder whether dietary guidelines can already be established since so many questions still need to be answered. It is likely that in the next decade more detailed information will become available. However, the role dietary fat, calorie intake, fibers, some vitamins and Se seem to play in tumor promotion allow some consideration of dietary guidelines, despite limited knowledge. The guidelines may not be very detailed and quantitative, but they may exert a certain impact on cancer occurrence. The proposed dietary guidelines of the USA (38), the Consensus Statement on Provisional Dietary Guidelines of the ECP (39), and those of the Dutch Nutrition Council (40) are tempting examples. These guidelines are designed to serve not only as a means to reduce cancer but also to reduce other morbidities such as cardiovascular disease, diabetes, adiposity, and other health threatening diseases. In general these guidelines are very similar: they recommend a decrease in fat intake, a varied diet with a variety of different vegetables and fruits, increase in complex carbohydrates (starch and fiber), avoidance of too much sugar, adequate intake of vitamins and minerals, moderation of alcohol intake, reduction in salt intake, avoidance of protein pyrolysates and maintenance of appropriate body weight.

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SUMMARY

This thesis deals with experimental investigations on the modifying effects of dietary factors on NMU-induced mammary carcinogenesis in female F344 rats. The goals of this study were essentially twofold:

(1) to test the hypothesis that dietary fat and protein influence development of NMU-induced mammary carcinogenesis in rats, and

(2) to assess whether these influences are mediated by altered hormone levels of prolactin, estradiol-17β, progesterone, corticosterone, and/or by altered steroid hormone receptors in mammary tumor cytosol.

The studies included 8-9 months of carcinogenicity bioassays. The experimental design was developed in a fashion allowing a systematic approach to determine interrelationships between dietary constituents (fat, linoleic acid {LA} and protein), hormones and NMU-induced mammary cancer. Therefore the general experimental design was of a factorial type. Groups of rats were fed diets that varied only in their content of the dietary component to be tested. After an adaptation period in which the experimental diets were fed mammary tumors were induced by just one intravenous injection of NMU (50 mg/kg body weight). Tumor response, growth, food intake, body weight gain, food conversion efficiency, plasma hormone levels, and steroid hormone receptor levels in tumor tissues in the various groups were parameters for estimating the modifying effect of the dietary factor under investigation. The values of these parameters were compared with results published in previous studies.

In Chapter 2 the literature on epidemiological and experimental studies relating to nutrition, hormones and breast cancer is reviewed. Possible mechanistic explanations are discussed. It is concluded that there is much information suggesting direct or indirect involvement of dietary fat in mammary carcinogenesis. The influence of either net energy intake, dietary protein, vitamins and minerals in general needs further substantiation, although moderate influence of those factors seems to be plausible. There is a need for further study to better understand the relationships between diet, hormones and breast cancer.

In Chapter 3 the influence of content and type of dietary fat and of the method of sacrifice on plasma hormone levels in healthy cyclic female F344 rats is described. The studies were undertaken to obtain more detailed information on the influence of content and type of dietary fat and of ether anesthesia during blood sampling on plasma levels of prolactin, estradiol, progesterone and corticosterone. It is shown that content and type of dietary fat from sources also used for human consumption (lard or sunflower seed oil) did not affect plasma levels of prolactin and estradiol. However, progesterone levels during proestrus, were higher in rats fed diets high and lower in rats fed diets low in sunflower seed oil, both as compared with rats fed high- and low-lard diets. Sunflower seed oil was associated with higher levels of corticosterone than lard, irrespective of the fat content and of the stage of the estrus cycle. Large effects of ether anesthesia were found as compared to decapitation after adaptation to the handling procedure. These effects varied largely during the various stages of the estrus cycle, and were particularly marked during metestrus-diestrus. These results suggest that differences in plasma levels of prolactin and of some steroid hormones, as reported in several publications and thoroughly discussed in Chapter 2, are likely to be due to insufficiently standardized experimental procedures, including handling and manipulation of the animals, rather than to either content or type of fat. This cycle dependent effect should be taken into account if considering the role of hormones in chemically induced mammary carcinogenesis. The data also stress the importance of handling habituated animals, of avoidance of anesthetics and of accurate confirmation of the stage of the estrus cycle, when administering the carcinogen and/or measuring the effect of diet on hormone levels.

Chapter 4 presents a systematic investigation of the interaction between LA content and total fat content in the diet in mammary tumor promotion. Beef tallow and safflower oil were used as the sources of fat to obtain the required LA and total fat contents of the diet. A 3x3 factorial design was chosen to compare low versus moderate and high contents of either dietary fat or LA content of the diet. Using multivariate and regression analysis we were able to analyze individual and interactive effects. It was shown that increasing the content of either fat or LA did not markedly influence tumor incidence. Increasing the LA content apparently resulted in increased tumor multiplicity, although not statistically significantly. Increasing the content of both fat and LA in the diet resulted in a non-significantly shortened time

to first tumor appearance and in a higher tumor yield. We concluded that a high LA content of the diet was associated with a tendency of a higher mammary tumor yield, although this effect needs confirmation. LA as such did not appear to affect mammary tumor incidence as does total amount of fat in the diet. From our study there appears to be no treshold of LA with respect to its enhancing effect on mammary carcinogenesis for dietary levels between 0.85 and 5.31% w/w (1.70-12.53% of energy). Rats that ultimately developed tumors grew faster in the last week before NMU application than animals that did not develop tumors. Rats in diestrus at the time of NMU exposure were more prone to develop tumors than animals in the other stages of the estrus cycle at that time, thus stressing the importance of cell multiplication in determining its vulnerabilty to carcinogens.

Chapter 5 describes a study on the interactive effects of dietary LA and total fat content of the diet on one hand, and plasma levels of prolactin, estradiol, progesterone and corticosterone on the other hand in relation to mammary carcinogenesis in a 3x3 factorial design. At the end of the study estrogen- (ER), progesterone- (PR) and androgen receptor (AR) levels in the tumors were estimated simultaneously in the same sample. It appeared that the level of fat, but not the LA content in the diet influenced the plasma levels of prolactin and estradiol. However, it was not a straight forward relationship. The effect of fat varied at different levels of LA. At 0.85% and 2.12% LA in the diet the highest levels of prolactin were observed in animals fed 17.5% fat in their diets, but at 5.31% LA the prolactin levels slightly increased with increasing fat content of the diet. Plasma estradiol levels increased with increasing fat content of the diet from 10 to 17.5%, but decreased at a further rise of the fat content to 25% at every LA level. A non-linear effect of LA, but no effect of fat, on plasma progesterone was found. Animals fed 2.12 % LA had significantly lower progesterone levels than animals fed either 0.85% or 5.31% LA. Plasma levels of corticosterone appeared not to be influenced by either dietary LA or dietary fat content. No differences between hormone levels of tumor-bearing and non-tumor-bearing animals were observed. Neither content nor affinity of ER, PR and AR in NMU-induced mammary tumors were influenced by dietary fat content. However, the LA content of the diet seemed to influence PR content. The AR content was inversely related to the lifespan of a tumor. The PR content and the affinity constants of ER and PR decreased with increasing weight of a tumor. It is concluded that, although certain influences of dietary fat and LA on endocrine parameters in

NMU-induced rat mammary carcinogenesis were observed, it is not likely that fat or LA intake per se results in clear effects on the biochemical action of prolactin, estradiol, progesterone or corticosterone. Additionally, it is more likely that the level of steroid hormone receptors in chemically induced mammary carcinomas is primarily dependent on the endocrine status of the animal rather than on dietary macronutrients such as dietary fat if fed at an adequate level. However, animals fed low to marginal or even deficient levels of dietary fat may exhibit reduced hormone levels in comparison with animals fed HF diets, most likely as a result of deficient or marginal fatty acids supplied by the low fat diets. There seems to be sufficient evidence now to conclude that reported increased prolactin levels in tumor-bearing animals are most likely the result of stress-inducing manipulation of the animals rather than a direct result of the diet consumed.

Chapter 6 reports the interactive effects of type and content of dietary protein on plasma prolactin and estradiol-17β and on tumor development in the NMU model. The effects of three levels of casein (animal protein) and soy protein isolate (vegetable protein), and mixtures of both proteins (80/20, 50/50, 20/80) at a relatively high constant level of fat (17.5%) in the diet were studied in detail. Protein levels were 15%, 22.5% and 30% w/w. Plasma levels of prolactin and estradiol-17β were assessed in tumor-bearing animals, while ER and PR levels were estimated simultaneously in the same tumor sample. Increasing the content of protein in the diet, at this high level of fat did not influence tumor incidence, but the lowest number of tumors was observed in animals fed soy protein isolate only. As in the study on LA and fat content of the diet (Chapter 5), tumor development was found to be related to the stage of the estrus cycle at the time of exposure to NMU. Additionally, tumor multiplicity was dependent on both the stage of the estrus cycle at induction and the type of protein consumed. Increasing the proportion of vegetable protein at the expense of animal protein was accompanied by a lowered tumor multiplicity and by a longer tumor latency. Dietary protein influenced plasma prolactin levels not unambiguously. Plasma prolactin levels in animals fed either 100% casein or 100% soy protein isolate were lower than those in animals fed any of the mixtures (80/20, 50/50, 20/80) tested. Plasma levels of estradiol-17β were not affected by either type or content of dietary protein. Tumors of animals fed casein had higher mean ER and PR receptor levels than those of animals fed soy protein isolate, which may indicate that there is a limiting amino acid in rations containing only soy protein isolate. Growth

velocity at the moment of initiation seemed to be an important determinant of consequent tumorigenesis.

GENERAL CONCLUSION:

The relationship between diet and mammary cancer is a very complex one. Diet directly influences the development of tissues, and therefore it can play an important role in chemical carcinogenesis. Diets, deficient or marginal in essential fatty acids or essential amino acids, most likely lead to pituitary insufficiency, decreased secretion of anterior pituitary hormones and decreased estrogen production. At the same time growth will be retarded, including development of mammary epithelium. The developing mammary gland responds a.o. to systemic hormonal stimuli of prolactin and estrogens. Estrogens and prolactin induce mitosis in mammary epithelial cells. By promoting cellular replication and DNA synthesis, these hormones sensitize the mammary gland to carcinogen binding. The multistep process of carcinogenesis, including the reaction of a normal cell genome with a genotoxic agent may result in an irreversible change in DNA, generally referred to as initiation. As yet unknown stimuli (probably particular growth factors), however, may promote the proliferation of preneoplastic cells, a process which may take several substeps of which some are reversible. However, only under favorable conditions the steps from initiation to progression of preneoplastic cells may be taken succesfully. Thus, both sufficiently developed anatomic structures, which are susceptible to carcinogenic stimuli (most likely the teminal end-buds) and a well developed endocrine system are needed to initiate carcinogenesis. In the consequent tumor promotion, both hormones and diet (most evidently dietary fat and energy consumption) have a certain stimulating role, but it is unlikely that dietary factors in adequately fed animals will affect tumorigenesis through influences on plasma levels of steroid hormones or prolactin.

SAMENVATTING

Dit proefschrift behandelt experimenteel onderzoek naar het modificerend effect van voedingsfactoren op door NMU geinduceerde mammatumoren bij vrouwelijke F344 ratten. De bedoeling is tweeledig:

(1) het toetsen van de hypothese dat vet en eiwit de ontwikkeling van door NMU geinduceerde mammatumoren bij ratten beinvloeden, en

(2) het bekijken of deze invloed verloopt via veranderde plasmagehalten van prolactine, oestradiol-17β, progesteron, corticosteron, en/of door veranderde receptoren voor steroid hormonen.

De onderzoekingen betreffen 8-9 maanden durende carcinogeniteit studies. De proefopzet is zo gekozen dat de relatie tussen voedingsstoffen (vet, linolzuur en eiwit), hormonen en door NMU geinduceerde mammatumoren systematisch onderzocht kan worden. Derhalve werd voor een factoriele opzet gekozen. Aan groepen ratten werden rantsoenen gevoederd die slechts van elkaar verschilden in het gehalte aan de component die onderzocht werd. Na een aanpassingsperiode waarin de proefrantsoenen gevoederd werden, kregen de dieren één injectie met NMU (50 mg/kg lichaamsgewicht). De tumorrespons, inclusief latentietijd en multipliciteit, groei van de dieren, voedselopname, efficientie van voedsel benutting, plasma hormoonwaarden en steroid-hormoon-receptor gehalten in tumor weefsel zijn de parameters waaraan mogelijke effecten gerelateerd worden. De resultaten werden vergeleken met resultaten van in de literatuur gerapporteerde studies.

In hoofdstuk 2 wordt een overzicht gegeven van de literatuur van epidemiologisch en experimenteel onderzoek betreffende voeding, hormonen en borstkanker. Mogelijke werkingsmechanismen worden bediscussieerd. De conclusie is dat er voldoende argumenten zijn om te veronderstellen dat vet zowel direkt als indirekt effekt kan hebben op het ontstaan van borstkanker. De invloed die energie opname, eiwit, vitaminen en mineralen zouden kunnen hebben dient nader onderbouwd te worden.

In hoofdstuk 3 wordt de invloed van zowel hoeveelheid als type voedingsvet, als ook van de methode van opofferen op plasma hormoonspiegels in gezonde cyclische vrouwelijke F344 ratten beschreven. Bovendien wordt de invloed van

ethernarcose op plasmaspiegels van prolactine, oestradiol, progesteron en corticosteron gedurende het verzamelen van bloed bestudeerd. Aangetoond werd dat zowel het gehalte als het type vet (dat ook voor menselijke consumptie gebruikt wordt, nl., varkensvet en zonnebloemolie) geen invloed hebben op plasma waarden van prolactine of oestradiol. Echter, gedurende de proestrus waren de progesteron waarden van dieren die hoge gehalten aan zonnebloemolie in het rantsoen hadden hoog, en die van dieren met lage gehalten aan zonnebloemolie laag, in vergelijking met de dieren die respectievelijk hoge of lage gehalten aan varkensvet in hun rantsoenen hadden. Hogere gehalten aan corticosteron werden waargenomen bij dieren die zonnebloemolie nuttigden, onafhankelijk van het vetgehalte en niet anders in de verschillende fasen van de oestrische cyclus. Ethernarcose bleek een duidelijk effekt te hebben in vergelijking met decapitatie na gewenning aan de procedure. Deze effekten waren wisselend in de verschillende fasen van de cyclus en het meest opvallend gedurende de metestrus-diestrus fase. Deze resultaten suggereren dat de verschillen in plasmaspiegels van prolactine en van sommige steroidhormonen, zoals in de literatuur gerapporteerd en zoals besproken in hoofdstuk 2, waarschijnlijk het gevolg zijn van onvoldoende gestandaardiseerde procedures, inclusief het manipuleren van de dieren, en niet van hoeveelheid of type vet in het rantsoen. Bij onderzoek naar de relatie 'hormonen en kanker' dient de onderzoeker hier wezenlijk rekening mee te houden. De resultaten benadrukken de noodzaak van het gebruik van handtamme dieren, het vermijden van ethernarcose en het zorgvuldig vaststellen van de fase van de cyclus.

In hoofdstuk 4 wordt de interaktie tussen gehalte aan linolzuur en gehalte aan vet in de rantsoenen systematisch onderzocht. Rundervet en saffloer-olie werden gebruikt om de gewenste gehalten linolzuur en totaal vet te bereiken. Een 3x3 factoriele opzet was gekozen om zowel lage, gematigde en hoge gehalten met elkaar te kunnen vergelijken. Met zowel multi-variate variantie- als regressie analyse konden zowel individuele als interactieve effecten worden getoetst. Noch een toename van vet, noch een toename van de hoeveelheid linolzuur in het rantsoen beinvloedde de tumorincidentie. Toename van de hoeveelheid linolzuur leidde ogenschijnlijk tot een verhoging van de tumormultipliciteit. Dit kon statistisch niet bevestigd worden. Een toename van zowel linolzuur als van vet ging gepaard met een niet significant verkorte latentietijd en een groter totaal aantal tumoren. Geconcludeerd werd dat een hoog gehalte aan linolzuur in het rantsoen gepaard ging met een tendens naar

meer tumoren, hoewel dit nader bevestigd dient te worden. Op basis van de resultaten van deze studie kan geen drempelwaarde voor een effect van linolzuur geidentificeerd worden voor gehalten tussen 0.85% en 5.31% w/w (1.70-12.53 energie %). Echter, ratten die tumoren kregen bleken sneller te groeien in de laatste week voor de NMU injectie. Bovendien bleken ratten in diestrus meer gevoelig voor het ontwikkelen van tumoren dan de soortgenoten in de andere stadia van de cyclus ten tijde van toediening van het carcinogeen.

In hoofdstuk 5 wordt de interaktie tussen gehalte aan linolzuur en gehalte aan vet in de rantsoenen systematisch onderzocht in relatie tot plasmaspiegels van prolactine, oestradiol, progesteron en corticosteron. De proefopzet is zoals in hoofdstuk 4. Bovendien worden aan het einde van de studie zowel oestrogeen- (ER), progesteron- (PR) en androgeen-receptor (AR) gehalten in tumoren bepaald, in hetzelfde cytosol. Het bleek dat wel het gehalte aan vet, maar niet het gehalte aan linolzuur invloed had op zowel plasma prolactine als oestradiol. Echter, dit was geen eenduidige invloed. Het effect van vet was wisselend voor verschillende gehalten aan linolzuur. Bij 0.85% en 2.12% werden de hoogste prolactine waarden gevonden in dieren die tevens 17.5% vet in hun rantsoen hadden. Bij 5.31% linolzuur nam het prolactine gehalte enigszins toe met toenemend vetgehalte in het rantsoen. Plasma oestradiol spiegels namen toe bij vetgehalten van 10% en 17.5% maar namen af bij een verdere toename van het vetgehalte tot 25% op ieder niveau van linolzuur. Vet beinvloedde plasma progesteron spiegels niet, maar dieren met 2.12% linolzuur in hun rantsoen hadden significant lagere progesteron spiegels dan dieren met 0.85 of 5.31% linolzuur in hun rantsoen. Plasma corticosteron spiegels werden niet beinvloed door vet of door linolzuur. Plasma hormoonwaarden van tumor-dragende dieren bleken niet te verschillen van die van dieren die vrij waren van mammatumoren. Noch de gehalten, noch de affiniteit van ER, PR, of AR in de tumoren werd beinvloed door vet in het rantsoen. Opmerkelijk was dat het gehalte aan AR omgekeerd evenredig was met de leeftijd van tumoren. Geconcludeerd werd dat, hoewel enige invloed van zowel vet als linolzuur op endocrine parameters in door NMU geinduceerde mammatumoren werd waargenomen, het niet waarschijnlijk is dat vet- of linolzuur-consumptie per se resulteert in een duidelijke invloed op de biochemische aktie van prolactine, oestradiol, progesteron of corticosteron. Het is aannemelijker dat het gehalte aan steroid-hormoon-receptoren in chemisch geinduceerde mammatumoren veeleer afhankelijk is van de endocriene status van het dier in kwestie dan van voedingsstoffen zoals vet, wanneer dat adequaat in het voer aanwezig is. Echter, dieren die deficiente of marginale

voeding consumeren kunnen waarschijnlijk wel lagere plasma-hormoonwaarden hebben dan adequaat gevoederde dieren, ten gevolge van het ontbreken van essentiele vetzuren. Er lijkt thans voldoende duidelijkheid te bestaan om te concluderen dat de hogere plasmaspiegels van prolactine in tumor dragende dieren het gevolg zijn van stress opwekkend behandelen van de dieren en niet het gevolg zijn van het rantsoen dat gegeten wordt.

Hoofdstuk 6 behandelt onderzoek naar interactieve effecten van zowel hoeveelheid als type eiwit op plasmaspiegels van prolactine en oestradiol en op de ontwikkeling van tumoren in het NMU model. De effekten van caseine (dierlijk eiwit) en soya-eiwit-isolaat (plantaardig eiwit), en van mengsels van deze beide eiwitten (80/20, 50/50, 20/80) op 3 niveau's (15%, 22.5% en 30 gewichts %) en op een relatief hoog vetgehalte (17.5%) werden bestudeerd. Een verhoging van het eiwitgehalte op dit vetniveau leidde niet tot een veranderde tumor incidentie. Echter, het minste aantal tumoren werd gezien bij dieren die alleen soya-eiwit-isolaat als eiwitbron hadden. Evenals in de studie naar de invloed van linolzuur en vet, bleek ook nu dat de ontwikkeling van tumoren gerelateerd was aan de fase van de cyclus ten tijde van toediening van het carcinogeen. De tumor-multipliciteit bleek afhankelijk van zowel de fase van de cyclus als van het type eiwit dat gegeten werd. Een toename van de hoeveelheid plantaardig eiwit, ten koste van dierlijk eiwit ging gepaard met een lagere tumor-multipliciteit en een langere latentietijd. Effekten op plasma prolactine spiegels waren niet eenduidig. De gehalten in plasma van dieren die of 100% caseine of 100% soya eiwit-isolaat als eiwitbron in hun rantsoen hadden, waren lager dan die van de dieren op elk der mengsels. Oestradiol spiegels vertoonden geen relatie met type of hoeveelheid eiwit in het rantsoen. De receptor-gehalten in tumoren van dieren die alleen caseine als eiwitbron hadden waren hoger dan die van dieren op soya eiwit-isolaat. Dit zou een aanwijzing kunnen zijn dat een aminozuur in het laatstgenoemde eiwit beperkend is. De groeisnelheid der dieren op het moment van blootstelling aan de carcinogene stof blijkt een belangrijk criterium voor het ontstaan van tumoren.

SLOT CONCLUSIE:

De relatie tussen voeding en borstkanker is van complexe aard. Voeding heeft een direkte invloed op de ontwikkeling van weefsels, en kan derhalve een belangrijke rol spelen in de chemische carcinogenese. Rantsoenen die deficient

of marginaal zijn in essentiele vetzuren of essentiele aminozuren, kunnen leiden tot een onvoldoende funktioneren van de hypofyse, leidend tot een verminderde secretie van hormonen en dus ook tot een verminderde produktie van oestrogenen. Tegelijkertijd zal de groei vertraagd zijn, inclusief de ontwikkeling van de epitheelcellen van de mamma. De zich ontwikkelende borstklier reageert o.a. op systemische hormonale prikkels van zowel prolactine als oestradiol. Oestradiol en prolactine induceren celdeling in epitheelcellen van de mamma. Door het stimuleren van celdeling en de synthese van DNA maken deze hormonen de borstklier gevoelig voor interaktie met carcinogenen. Het meerstapsproces van de carcinogenese, inclusief de reaktie van een normaal cellulair genoom met een genotoxisch agens zou kunnen resulteren in een onomkeerbare verandering van DNA. Dit wordt in het algemeen beschouwd als initiatie. Momenteel nog niet nader geidentificeerde stimuli (mogelijk specifieke groeifaktoren) kunnen preneoplastische cellen aanzetten tot proliferatie, een proces dat meerdere tussenstappen kan hebben en waarvan er enkele omkeerbaar kunnen zijn. Echter, alleen onder gunstige condities kunnen de stappen van initiatie tot progressie van preneoplastische cellen succesvol genomen worden. Derhalve moeten dus voldoende ontwikkelde anatomische strukturen (mogelijk de 'terminal end-buds'), die gevoelig zijn voor carcinogene prikkels maar ook een voldoende ontwikkeld endocrien systeem, aanwezig zijn om de carcinogenese te kunnen initieren. Bij de latere promotie kunnen hormonen en voeding (het meest duidelijk zijn vet en energie consumptie) een zekere stimulerende rol vervullen. Het is onwaarschijnlijk dat voedingsfactoren in adequaat gevoederde dieren het proces van de carcinogenese beinvloeden via plasmaspiegels van steroidhormonen of prolactine.

CURRICULUM VITAE

Bert Bunnik werd 1 januari 1953 geboren te Nijmegen en doorliep de lagere school aldaar. In 1972 werd het gymnasium B diploma behaald te Apeldoorn aan het gymnasium van het aartsbisschoppelijk seminarie. In datzelfde jaar werd de studie medische biologie (B5*) aan de Rijksuniversiteit Utrecht aangevangen. Het kandidaatsexamen werd in 1977 behaald. Het doctoraalexamen werd in 1980 behaald met als hoofdvakken Vergelijkende Endocrinologie (Prof.Dr. P.G.W.J van Oordt) en Humane Fysiologie (Prof. P.A.Biersteker) em met als bijvak Medische Enzymologie (Prof.Dr. G.E.J.Staal).

Tijdens de studieperiode werd intensief aan sport gedaan en werden diverse sportkaderopleidingen gevolgd en succesvol afgesloten. Medewerking wordt verleend aan de kaderopleidingen van de KNHB (Koninklijke Nederlands Hockey Bond). Tussen 1980 en 1988 werden diverse buitenlandse stages gevolgd en verzorgd. In 1983 werd door de FIH (Féderation Internationale de Hockey) een benoeming tot internationaal trainer verleend. Dit leidde o.a. tot deelname aan de Olympische Spelen van 1984 te Los Angeles, als coach van de Spaanse herenploeg.

Na het voltooien van de universitaire opleiding werd van 1980-1983 een KWF (Koningin Wilhelmina Fonds) fellowship toegekend (mentor:Prof.Dr. H. Bloemendal). Tijdens deze periode werd de praktische vaardigheid in de experimentele carcinogenese verworven bij de afdeling Biologische Toxicologie van het Instituut CIVO-Toxicologie en Voeding TNO, te Zeist (Hoofd: Dr.V.J.Feron). Tevens werd de kennis vermeerderd op de gebieden proefdierkunde, pathologie, chemische endocrinologie (met name de androgene steroidhormonen) en chemische carcinogenese (IARC, Lyon). Een onderzoeksvoorstel werd geschreven over de relatie Voeding-Hormonen-Borstkanker en ter subsidiering aangeboden aan het KWF. Dankzij de subsidie van het KWF en de bijdrage van de Hoofdgroep Voeding en Voedingsmiddelen TNO, kon een dienstverband worden aangegaan met TNO. Sedert 1983 is de schrijver van dit proefschrift in dienst van de Nederlandse Organisatie voor Toegepast Natuurwetenschappelijk Onderzoek TNO. Van 1 maart 1983 tot 1 april 1988, als wetenschappelijk medewerker van de afdelingen Biologische Toxicologie (Hoofd: Dr.V.J.Feron) en Klinische Biochemie (Hoofd: Prof.Dr.W.H.P.Schreurs). Het in dit proefschrift beschreven onderzoek werd uitgevoerd op de afdeling Biologische Toxicologie van het Instituut CIVO-Toxicologie en Voeding TNO, te Zeist tussen 1983 en 1988. In 1984 werd het diploma "Deskundigheid Stralingsbescherming" behaald te Leiden. Sedert 1 april 1988 is betrokkene Staflid Onderzoeksbeleid van de Hoofdgroep Gezondheidsonderzoek-TNO, te Rijswijk.

krips repro meppel