

Dietary Protein, Enhancement of *N*-Nitrosomethylurea-induced Mammary Carcinogenesis, and Their Effect on Hormone Regulation in Rats¹

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ABSTRACT

The effect of supplemental dietary protein (casein) fed with high fat diets was investigated using the *N*-nitrosomethylurea-induced mammary tumor model. Isocaloric diets containing casein and corn oil at 19 and 15% (normal protein-high fat) or 33 and 15% (high protein-high fat) were fed *ad libitum* to Sprague-Dawley mother rats. Female offspring continued on the diet. Food consumption and growth curves were similar over the entire growth period. *N*-Nitrosomethylurea (50 mg/kg body weight) or saline was administered at 7 and 8 weeks of age via the tail vein. Dietary protein had no effect on serum prolactin or growth hormone throughout the estrous cycle: Prior to carcinogen administration, at 7 weeks old, proestrus at 5 p.m., serum prolactin was 231.6 ± 141.0 (SE) ng/ml (12 rats) versus 292.2 ± 141.0 (13 rats) for normal versus high protein diet groups, respectively. No difference was noted after carcinogen injection at 9, 13, 28, and 33 weeks of age. Similarly no effect was noted on serum growth hormone activity.

Tumor latency was 7 weeks and incidence was 100% in normal protein (24 rats) and high protein (39 rats) groups 28 weeks after carcinogen treatment. The number of tumors per rat (4.38 ± 0.37 versus 2.87 ± 0.35 , $P < 0.002$) and average tumor weight (17.97 ± 2.63 versus 9.94 ± 2.92 g) were significantly greater in the high protein group. Study indicates that diet or carcinogen treatment did not alter hormone regulation during the estrous cycle. However, supplemental dietary protein increased the effect of high fat diets enhancing the mammary tumor burden.

INTRODUCTION

Epidemiological studies suggest that dietary protein may enhance breast tumor growth either independently or synergistically with dietary fat (1). Dietary studies with animals are limited and primarily used the procarcinogen DMBA³ for induction of mammary tumors. Clinton *et al.* (2) demonstrated that following weaning, increased dietary casein during the preinitiation period resulted in a decreased mammary tumor incidence. Dietary protein, however, did not influence the promotional phase of tumor growth. We have reported (3) that increased dietary protein fed over a lifetime enhanced DMBA mammary tumor incidence. Recently Clinton *et al.* (4) examined the interaction of dietary protein and fat fed after weaning on DMBA-induced mammary tumors. These studies demonstrated an increasing tumor incidence with increased corn oil consumption. However, no effect from dietary protein or protein-fat interaction was observed.

NMU, a direct carcinogen with a short half-life, induces mammary adenocarcinoma (5). Animal studies by Dao *et al.* (6, 7), demonstrated that increased dietary fat enhances NMU-induced mammary tumors. Further studies established oleic

and linoleic acid as the constituents responsible for enhancement and that saturated medium chain triglycerides reduced the effect (8). The effect of the high fat diet on mammary tumor enhancement occurs during the age of maximum mammary gland development, approximately 50 days of age (9).

Studies by Arafah *et al.* (10) and Rose and Noonan (11) demonstrated that the NMU-induced mammary tumors were dependent on estrogen, prolactin, and to a lesser degree growth hormone for growth.

Utilizing the direct short lived carcinogen, NMU, this study reports on the effect of dietary protein and protein-fat on the enhancement of mammary tumor growth. The report also examines the effect of diet and NMU administration on hormone regulation.

MATERIALS AND METHODS

Diets and Feeding Schedule. A previously published (3) feeding protocol was followed. Adult virgin Sprague-Dawley rats (Harlan, Madison, WI) were housed in a temperature-controlled room ($24 \pm 2^\circ\text{C}$) with 14 h of illumination. During the initial 2-week conditioning period the rats were fed Purina rat chow (Ralston Purina Co., St. Louis, MO) *ad libitum*. The animals were subsequently fed the NP-HF or HP-HF isocaloric diets *ad libitum* for 3 weeks. The test diets were continued through mating, gestation, and lactation. On the day of birth, litters were sexed and reduced to eight pups. After weaning (21 days) all female pups were continued for the entire experiment on the test diet consumed by their mother. Ten rats, grouped in pairs, on each test diet were followed weekly for 30 weeks to determine food consumption and growth curve. Waste food was carefully removed and weighed.

Diet composition, listed in Table 1, was prepared as pellets by ICN Nutritional Biochemicals, Inc., Cleveland, OH, and stored at 4°C .

Tumor Induction and Assessment. At 7 and 8 weeks of age each rat received a 50-mg/kg body weight dose of NMU (ICN Pharmaceutical, Inc., Plainview, NJ). NMU was administered i.v. via the tail vein under light ether anesthesia according to the procedure of Rose and Noonan (12); controls were given injections of saline.

The effect of NMU injection on the estrous cycle was determined by vaginal smear technique for six cycles immediately following injection.

Animals were examined weekly for palpable tumors. Rats were sacrificed 28 weeks after the second NMU injection and each mammary tumor was excised and weighed. Each rat was also examined for secondary lesions.

Hormone Assays. At scheduled intervals sequential blood samples (0.5 ml/rat) were collected by orbital sinus puncture under light ether anesthesia on 4 consecutive days at 10 a.m. and at 5 p.m. on the day of proestrus. Serum was stored at -20°C for hormone assay. Anterior pituitary tissue was obtained after sacrifice, homogenized in phosphate buffer (1 ml), diluted 40-fold, and assayed for prolactin and growth hormone.

Growth hormone and prolactin concentration were measured using the rat radioimmunoassay kit which was provided by the National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases. Prolactin levels were expressed in terms of the rat RP-3 standard, which has a biological potency of $2.8 \times$ Reference Preparation 1. Growth hormone levels were expressed in terms of the rat growth hormone-Reference Preparation 2 standard, which has a biological potency of $2.5 \times$ Reference Preparation 1.

On the first proestrous day (2 p.m.) following NMU or saline treatment, catecholamine synthesis was blocked by i.p. administration

Received 11/13/85; revised 3/20/86, 5/27/86; accepted 6/2/86.

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¹ This study was supported by Grant CA-35573 awarded by the National Cancer Institute, NIH, Bethesda, MD 20205.

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³ The abbreviations used are: DMBA, 7,12-dimethylbenz(a)anthracene; NMU, *N*-nitrosomethylurea; NP-HF, normal protein-high fat; HP-HF, high protein-high fat; α -MPT, α -methyl-*p* tyrosine; i.g., intragastric.

of the tyrosine hydroxylase inhibitor, α -MPT, 250 mg/100 g body weight (13). Animals were sacrificed 45 min later and prolactin and growth hormone concentrations were determined in serum and pituitary gland.

Statistical Analyses. Statistical differences in tumor incidence between test groups were determined by χ^2 analysis with Yates correction (14). The statistical difference in average latency period in tumor appearance, number of tumors per rat, body weight, and hormone activities between groups was determined by analysis of variance and Student-Newman-Keul's test (15).

RESULTS

The isocaloric diets were fed *ad libitum*. Food consumption was determined from weaning through 30 weeks of age. The data summarized in Fig. 1 indicate that animals feeding on either the HP-HF or the NP-HF diet consumed similar amounts of food. The two test groups had a similar growth curve as illustrated in Fig. 2.

At 7 weeks of age, prior to the administration of NMU, serum prolactin and growth hormone concentrations were determined throughout the estrous cycle. The data summarized in Fig. 3 indicate that diet had no effect on hormone release. Following the injection of NMU or saline, serum prolactin and growth hormone concentrations were determined at 13, 18, 28, and 33 weeks of age. The data summarized in Fig. 4 further substantiate the lack of any diet effect and also any effect of NMU or saline treatment on serum hormone activities throughout an estrous cycle 5 weeks after NMU administration. Similar results were obtained at 18, 28, and 33 weeks of age.

Animals were sacrificed at 2 p.m. on the first proestrous period following the second treatment with NMU or saline. Additional animals were treated with the tyrosine hydroxylase

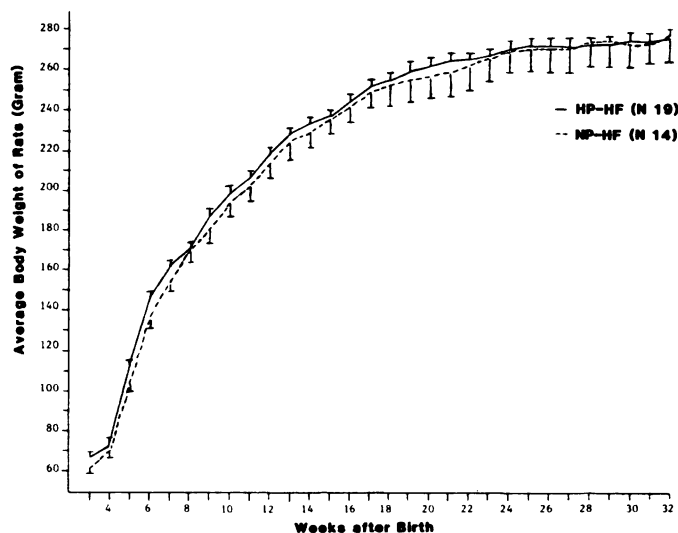


Fig. 2. Growth curve showing average body weight measured at weekly intervals for rats fed *ad libitum* isocaloric NP-HF or HP-HF diets. Bars, SE.

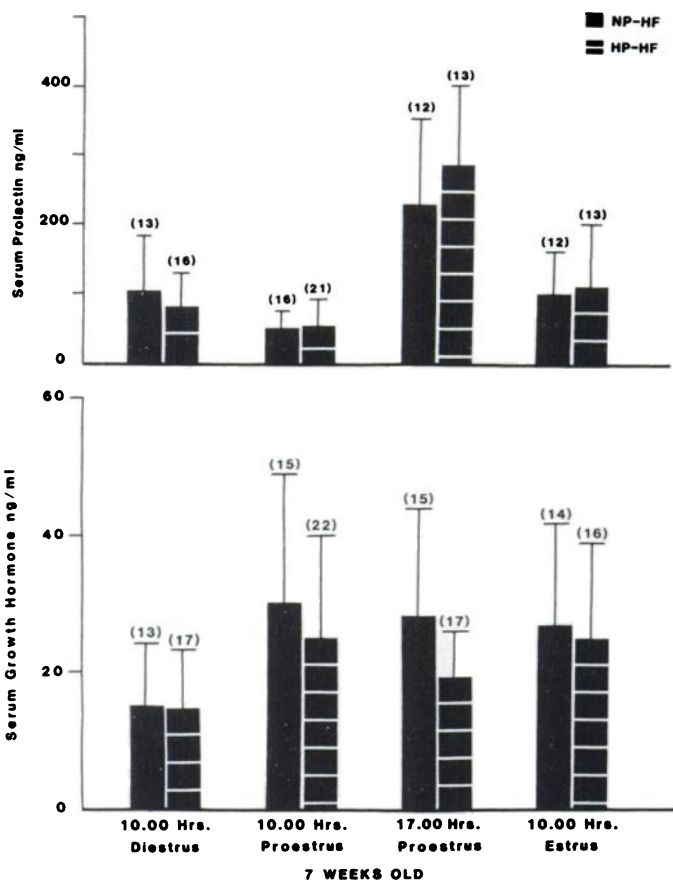


Fig. 3. Serum prolactin and growth hormone concentrations (ng/ml) measured at four stages of the estrous cycle in rats fed isocaloric NP-HF or HP-HF diets. Determinations made before NMU treatments. Numbers in parentheses, number of animals. Bars, SD.

Ingredients	Normal protein (19%), high fat (15%)	High protein (33%), high fat (15%)
Casein (vitamin free)	19	33
Corn oil	15	15
Corn starch	25	25
Sucrose	31	17
Fiber (Alphacel)	5	5
Mineral Mix (AIN)	3.5	3.5
Vitamin Mix (AIN)	1	1
Choline bitartrate	0.2	0.2
DL-Methionine	0.3	0.3

^a 4.35 kcal/g.

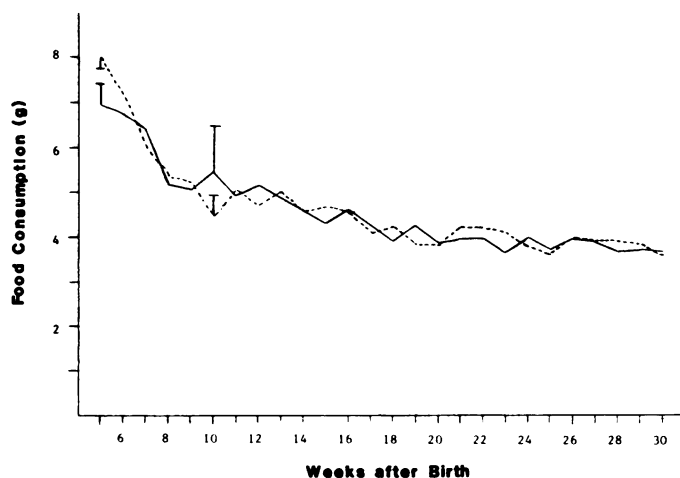


Fig. 1. Average daily food consumption per 100 g body weight measured at weekly intervals. There were ten rats in each diet group. Bars, SD; —, HP 33%, HF 15%; ---, NP 19%, HF 15%.

inhibitor α -MPT and sacrificed 45 min later. Prolactin and growth hormone levels were determined in the blood and pituitary gland. Inhibition of dopamine synthesis caused a significantly increased release of prolactin without significantly altering the concentration in the pituitary gland (Table 2). Immediately after NMU treatment the basal level or the release of prolactin was not influenced by diet or carcinogen.

Following α -MPT treatment serum growth hormone concen-

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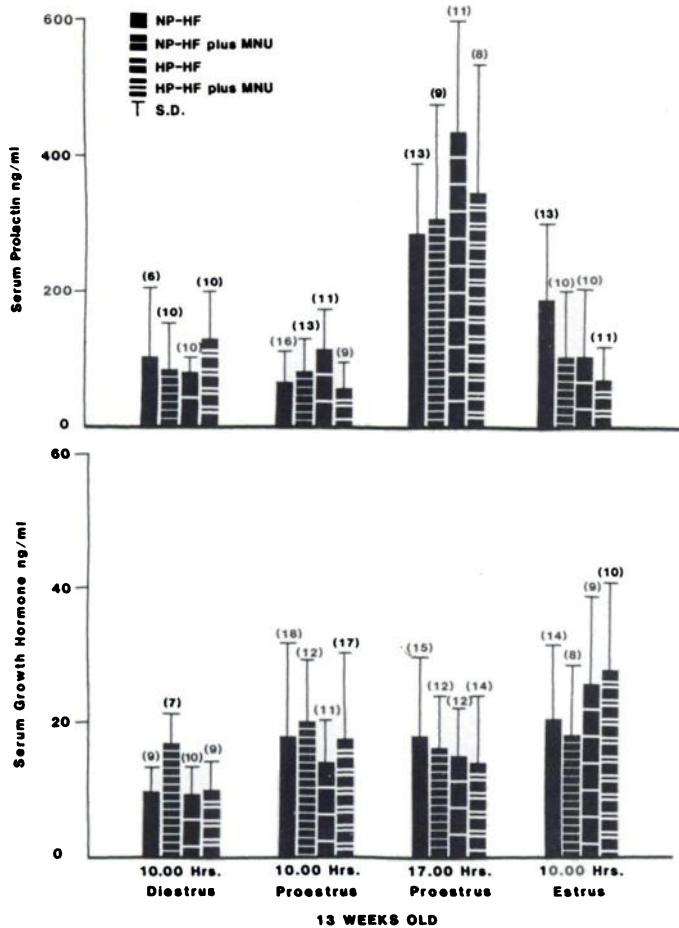


Fig. 4. Serum prolactin and growth hormone concentrations (ng/ml) measured at four stages of the estrous cycle in rats fed isocaloric NP-HF or HP-HF diets. Determinations made 5 weeks after NMU (NMU) (50 mg/kg body weight) or saline treatment. Numbers in parentheses, number of animals. Bars, SD.

Table 2. Serum and pituitary prolactin activity after α -MPT treatment following NMU administration

Treatment	NP-HF		HP-HF	
	Base ^a	α -MPT ^b	Base	α -MPT
Serum prolactin (ng/ml)				
Saline	50.3 \pm 32.2 ^c	169.2 \pm 128.0	27.5 \pm 5.6	204.0 \pm 138.0
NMU	27.5 \pm 8.7	153.2 \pm 65.3	45.1 \pm 26.2	145.2 \pm 27.0
Pituitary prolactin (μg/mg protein)				
Saline	72.7 \pm 26.0	44.5 \pm 10.9	58.1 \pm 6.7	41.1 \pm 14.2
NMU	46.8 \pm 7.8	55.0 \pm 13.7	61.8 \pm 8.7	41.6 \pm 10.0

^a Animals (5/group) sacrificed by decapitation; first estrous cycle (proestrus, 2 p.m.) after second NMU or saline injection.

^b Sacrificed 45 min after α -MPT treatment.

^c Mean \pm SE.

tration was decreased but the concentration remained essentially unchanged in the pituitary gland (Table 3). No significant effect was noted due to dietary protein or NMU treatment.

Following NMU treatment animals were examined daily for eight estrous cycles. The average length of cycle was 5 days which is equivalent to the cycle length prior to NMU treatment. In addition no differences were noted which related to dietary protein.

The first palpable tumor was evident in each diet group 7 weeks after the second dose of NMU was administered (Fig. 5). The number of animals with tumors increased equivalently in the two groups. All animals were tumor bearing 28 weeks after NMU administration.

Table 3. Serum and pituitary growth hormone activity after α -MPT treatment following NMU administration

Treatment	NP-HF		HP-HF	
	Base ^a	α -MPT ^b	Base	α -MPT
Serum growth hormone (ng/ml)				
Saline	15.2 \pm 4.0 ^c	3.0 \pm 2.4	11.4 \pm 6.4	2.2 \pm 1.3
NMU	19.6 \pm 12.2	3.2 \pm 2.0	17.0 \pm 12.9	3.9 \pm 2.3
Pituitary growth hormone (μg/mg protein)				
Saline	275.5 \pm 56.9	230.2 \pm 57.9	211.1 \pm 28.0	149.4 \pm 39.4
NMU	204.3 \pm 49.8	192.7 \pm 10.6	275.0 \pm 40.8	209.6 \pm 15.7

^{a, b, c} See footnotes to Table 2.

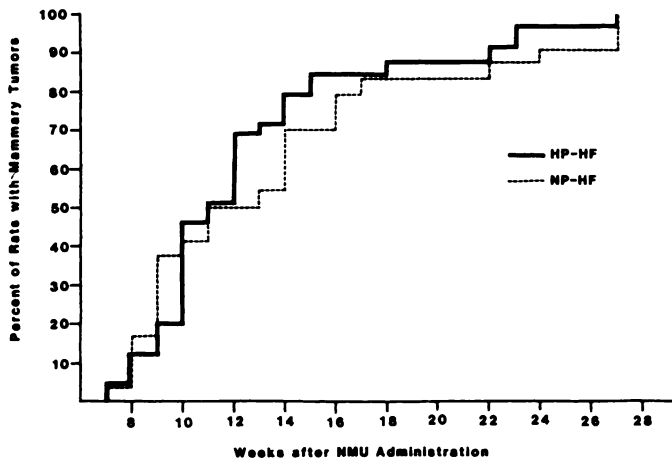


Fig. 5. Mammary tumor incidence in rats fed high fat (HF) diets mixed with either normal protein (NP) or high protein (HP) throughout their lifetime. NMU treatment (50 mg/kg body weight i.v.) at 7 and 8 weeks of age.

However, the HP-HF group had a significantly greater number of palpable tumors per rat compared to the NP-HF group (Table 4). In addition the average tumor weight was significantly greater, approximately 80%, in the HP-HF group.

At termination autopsies were performed on all animals; no metastatic lesions were observed at other organ sites.

DISCUSSION

Welsch *et al.* (16) reported that the prolactin-suppressing drug bromocriptine reduces significantly the number of rats with mammary tumors and the number of tumors per rat when administered after exposure to NMU. Arafah *et al.* (10) determined that 17 β -estradiol and prolactin worked synergistically to reactive tumor growth in hypophysectomized rats. Rose and Noonan (11, 12) demonstrated that regression of NMU-induced mammary tumors was halted or reversed after both prolactin and growth hormone replacement. Therefore NMU tumors are estrogen and prolactin dependent and are enhanced by growth hormone.

To establish whether the test diets may differentially affect hormone release and thereby influence tumor growth, serum prolactin and growth hormone concentrations were determined at 7, 13, 18, 28, and 33 weeks of age. As indicated in Figs. 3 and 4 the prolactin and growth hormone activities were essentially unchanged throughout the estrous cycle in the two diet groups at five different ages. Therefore differences noted in the tumor growth characteristics in this study cannot be explained by prolactin or growth hormone concentrations.

Kerdelhue and Abed (17) reported i.g. administration of DMBA at a dose of 15 mg/rat significantly inhibited luteinizing

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Table 4 Mammary tumor incidence 28 weeks following administration of NMU

Rats fed either a HP-HF or NP-HF isocaloric diet.

Treatment	No. of rats	No. of rats bearing tumors	Total no. of tumors	Av. no. of tumor/tumor-bearing rat	Av. wt of tumor/tumor-bearing rat (g)	Av. latency period (wk)
HP-HF						
Saline	24	0	0	0	0	0
NMU	39	39 ^a (100%)	171	4.38 ± 0.37 ^{b, c}	17.97 ± 2.63 ^c	12.58 ± 0.77
NP-HF						
Saline	25	0	0	0	0	0
NMU	24	24 ^a (100%)	69	2.87 ± 0.35	9.94 ± 2.92	14.0 ± 1.23

^a Pups derived from mothers on test diet; all female pups utilized on study protocol.^b Mean ± SE.^c Significant difference ($P < 0.002$).

hormone and follicle-stimulating hormone surges and stimulated prolactin surge on the afternoon of proestrus. Nine cycles after administration 70% of the animals experienced lengthened estrous cycles. It was concluded that DMBA affects hypothalamo-pituitary regulation. Utilizing a lower dose (5 mg/rat) of DMBA, we reported (18) that the prolactin, estrogen, and progesterone surge was normal in the first estrous cycle following treatment. We also observed 10 estrous cycles following DMBA administration and reported (19) that the estrous cycles remained unchanged. These data indicate that neurohormonal regulation is affected by DMBA at a high toxic dose and may influence tumorigenesis; however, at the lower dose no hormone effect is apparent.

The data in this study also indicated that NMU treatment had no immediate effect on neurohormonal regulation (Tables 2 and 3). Prolactin and growth hormone released from the pituitary gland was equivalent to saline-treated controls.

With either the NP-HF or the HP-HF diet the tumor latency period was the same (Fig. 5) and the NMU-induced tumor incidence was 100% in both groups 28 weeks after treatment. Rose *et al.* (20) utilizing the same NMU dose schedule with injection intervals of 1 week or 1 month demonstrated that latency was influenced by the length of the interim between doses. Utilizing single NMU injections, McCormick *et al.* (21) determined that latency and tumor incidence were related positively with NMU dose. In view of these observations, the 100% tumor incidence observed in this study would suggest that the dose of NMU should be reduced.

Clinton *et al.* (2) reported that increased dietary protein fed after weaning, prior to DMBA administration, decreased mammary tumor incidence and had no effect during the promotional phase. Recently Clinton *et al.* (4) reported on the interaction of dietary protein and fat on DMBA-induced mammary tumors. These studies indicated that fat correlated positively with tumor incidence; however, protein was ineffectual. These animals were fed the test diets after weaning and no difference in the age of sexual maturation was noted. This is in contrast to our model which begins *in utero*. Stern *et al.* (22) noted that prior to weaning protein diet can alter catecholamine concentration in the hypothalamic area and may relate to the early sexual maturation noted in our high protein animals (3). Clinton *et al.* (2) also indicated that the procarcinogen, DMBA, is more rapidly metabolized by the induced aryl hydrocarbon hydroxylase enzyme in the liver of animals fed a high protein diet. This would not be a contributing factor with the direct carcinogen, NMU, utilized in this study.

In this study the number of mammary tumors per rat was approximately 50% greater and the average tumor weight was 80% greater in the HP-HF group (Table 4). These data confirm the results previously reported (3) with the DMBA using a

similar animal model. Birt and Pour (23) recently reported that hamsters fed various fat-protein diets and treated with *N*-nitrosobis(2-oxopropyl)amine developed the largest numbers of neoplasms in the HP-HF group. An early study by Shay *et al.* (24) demonstrated a positive correlation with increasing dietary casein and 3-methylcholanthrene-induced mammary tumors. Ross *et al.* (25) demonstrated that in rats allowed to freely select their diet, the incidence of spontaneous neoplasms was correlated in part with high absolute protein intake shortly after weaning.

The age of sexual maturation is positively related to increasing dietary protein (3) when initiated over a lifetime. Early development of the breast in this study may relate to enhanced sensitivity to carcinogens and in part explain reported differences with test diets fed after weaning. These data demonstrate that supplemental dietary proteins can enhance the effect of high fat diets on the NMU-induced mammary tumor burden.

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